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INVESTIGATIONS ON MUMPS VIRUS

PROPAGATION OF MUMPS VIRUS IN CHICK EMBRYOS DURING SERIAL ALLANTOIC PASSAGES WITH UNDILUTED INOCULA

BY

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MERCATORIN KIRJAPAINO

to my wife

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Helsinki, January 1959.

Kari Cantell

CONTENTS

SURVEY OF THE LITERATURE 9

MUMPS VIRUS 9

Cultivation in Chick Embryos 9
— Short Historical Survey 9
— Effect of Virus on the Host 10
— Propagation in the Allantoic Cavity 11

Cultivation in Other Hosts 15

Virus-Red Cell Interaction 16
— Hemagglutination 16
— Hemolysis 18

Complement Fixing Antigens 18

Physical and Chemical Properties 19
— Structure 19
— Stability 19

»INCOMPLETE» VIRUSES 20

Influenza Virus 20
— von Magnus Phenomenon 20
— Production of Incomplete Virus 21
— Properties and Nature of Incomplete Virus 22

Other Viruses 23
— Newcastle Disease Virus (NDV) 23
— Fowl Plaque Virus 23
— Vesicular Stomatitis Virus (VSV) 24
— Rift Valley Fever Virus (RVF Virus) 24
— Miscellaneous Viruses 24

CONCLUSIONS FROM THE LITERATURE AND SCOPE OF THE PRESENT INVESTIGATION 25

PRESENT INVESTIGATION 27

Terms and Abbreviations Used 27

Material and Methods 27
Viruses 27
Eggs 28
Allantoic Passages 28
Storing of Virus Preparations 29
Red Cells 29
Hemagglutination Titrations 30
Infectivity Titrations 30
Complement Fixation Titrations 32
YIELD OF HEMAGGLUTININS AND COMPLEMENT FIXING ANTIGEN(S) DURING SERIAL UNDILUTED PASSAGES (UP-SERIES) AND STANDARD PASSAGES (ST-SERIES) 34
— Yield of Hemagglutinins and Complement Fixing Antigen(s) during UP- and ST-series with Incubation Time of Five Days in Each Passage 34
— Yield of Hemagglutinins during UP-series with Incubation Time of Two and Three Days in Each Passage 39
— Inhibitors of Hemagglutination in Allantoic Fluids from UP-series 41
Discussion 43
HEMAGGLUTININ TITRES IN ALLANTOIC FLUIDS OF INDIVIDUAL EGGS 45
— Egg Variation during UP- and ST-series 45
— Seasonal Variation in Yield of Hemagglutinins 50
Discussion 50
COMPARISON BETWEEN INFECTIVITY AND HEMAGGLUTININ TITRES DURING UP- AND ST-SERIES 52
— Growth Curve after Inoculation of Undiluted ST-seed 52
— Infectivity and Hemagglutinin Titres in UP- and ST-series with Incubation Time of Five Days in Each Passage 53
— Infectivity and Hemagglutinin Titres in UP-series with Incubation Time of Three Days in Each Passage 56
Discussion 50
INTERFERENCE PHENOMENA 61
— »Autointerfering» Activity of UP-virus Preparations.. 61
— Interfering Capacity of UP-virus Preparations and of Inactivated Virus 65
Discussion 68
GENERAL DISCUSSION 70
SUMMARY 77
REFERENCES 79

SURVEY OF THE LITERATURE

MUMPS VIRUS

CULTIVATION IN CHICK EMBRYOS

Short Historical Survey

Granata (1908) was probably the first to suggest that mumps is caused by a filtrable virus (62). In 1934 Johnson & Goodpasture (97) first succeeded in cultivating the virus continuously under laboratory conditions. They infected monkeys by injecting them via Stensen's duct with saliva from mumps patients and were able to pass the virus by inoculating infected parotid gland suspension into other monkeys. Some remarkable results were reached later with monkeys in experimental studies of mumps. Thus, using infected parotid gland material as antigen, Enders & Cohen (38) in 1942 developed a complement fixation test and Enders (39) in 1943 a skin test by means of which information could be obtained regarding the immunity of man and experimental animals to mumps virus.

However, the study of many questions related to mumps virus was extremely difficult in the monkey, and so it is understandable that, in the cultivation of mumps virus, investigators tried to use more suitable hosts, notably chick embryos, which had proved very well suited for many kinds of virological investigations in the 1930's and the early 1940's (59, 142, 15).

Rocchi (141), in 1943, was the first to report on successful cultivation of mumps virus in eggs. He inoculated different samples from mumps patients onto the chorioallantoic membranes of 8- to 12-day-old chick embryos and found changes in the membranes after incubation for 4 to 8 days; the changes, however, could never be observed during more than six passages. The lesions described

by Rocchi have later been regarded as nonspecific (155): similar changes have recently been described following inoculation of suspension of normal chorioallantoic membrane (178). It should be noted, however, that Rocchi once caused a parotid swelling fairly typical of mumps to arise even with membrane suspension from the sixth egg passage.

Meanwhile the attempts of other investigators to cultivate mumps virus in chick embryos failed (157, 40).

In 1945 it was definitely demonstrated by Habel (67) that the chick embryo is a susceptible host for the mumps virus. He inoculated parotid gland material from an infected monkey into the yolk sac of 5- to 7-day-old embryos or into the amniotic or allantoic cavity of 10-day-old embryos. After 7 to 12 days he was able to show the presence of virus by means of the complement fixation test in which an unknown fluid or suspension from embryos was titrated against a known positive convalescent serum. Habel also showed that, in chick embryos, it was possible to perform neutralization tests for demonstration of antibodies and that the egg antigen was suitable for use in the skin test. In the same year Levens & Enders (104) already confirmed these findings concerning mumps virus multiplication in embryonated eggs. In addition they made the important observation that the amniotic and allantoic fluids of infected eggs agglutinated fowl erythrocytes. After this, mumps virus preparations could thus be titrated not only by the CF test but also by hemagglutination methods identical to those earlier developed for titration of influenza virus preparations (82, 143).

Habel had failed in his two attempts to isolate mumps virus in chick embryos directly from human cases. In 1946, however, Beveridge *et al.* (7) reported that they had succeeded in isolating the virus from the saliva of mumps patients in the yolk sac and amniotic cavity of chick embryos. Isolated virus strains could also be passed allantoically.

Following these findings, chick embryos practically ousted monkeys as experimental animals in studies of mumps.

Effect of Virus on the Host

Using any of the different routes of inoculation, mumps virus has not been found to cause typical macroscopic or microscopic

lesions in 7- to 8-day-old chick embryos (67, 7, 168, 43). (In roller cultures of chorioallantoic membrane the Enders strain of mumps virus has given rise to cytoplasmic inclusion bodies (13)). Some of the embryos may die, but death of the embryo is an inconsistent and irregular phenomenon (43). Mortality has been reported to increase if a large inoculum is used (48). Studies of the fate of mumps virus in the chick embryo by the fluorescein-labelled antibody technique have proved that the virus is not invasive (168). Multiplication seems to be limited to the cells in contact with the infected fluid. The virus evidently is able to multiply in and be released from the cells without causing destruction (168). The sensitivity of chick embryos to mumps virus decreases with increasing age (172, 168, 111). The cells probably are either altered or replaced by less susceptible ones (168). In a 16-day-old embryo no multiplication of virus seems to occur any longer (168).

The nature of mumps infection in very young chick embryos differs from that seen in older embryos. The virus is lethal and causes cataracts and other defects in 2-day-old chick embryos (67a, 174).

Propagation in the Allantoic Cavity

Adaptation. — The amniotic cavity is the route of choice for isolation of mumps virus (105, 106, 99, 85, 69, 43, 161). The hemagglutination test is almost solely used as the criterion for infection. In the studies of some investigators, all the strains isolated already gave agglutination in the first passage (85). Others found that, in the case of some isolated strains, the amniotic fluid did not begin to show agglutinating activity until in the second, third or even fifth passage (105, 69, 161). For continued cultivation of isolated strains, allantoic passages have certain advantages over amniotic passages: the technique is simpler and a larger amount of infectious material is obtained. Ease of adaptation to the allantoic cavity varies from one strain to another (9, 172, 110, 154, 138). Adaptation of some strains to the allantoic cavity has failed in spite of many attempts (110, 138), propagation of some strains has remained irregular through numerous passages (9, 154), while some strains have grown fairly regularly since the first few passages (138). Hemagglutinins are produced during the adaptation phase in the

allantoic fluids of only some of the chick embryos (9, 69, 155). During several allantoic passages growth improves with the number of passages (9, 41). One of the mumps strains best adapted to the allantoic cavity is the Enders strain — the type strain of mumps virus (4) — which has been used in most studies dealing with the propagation of mumps virus in the egg.

Factors Affecting Yields. — Possible ways and means of obtaining as high virus titres as possible in the allantoic fluids have been studied chiefly with a view to the preparation of antigens and vaccines. Attention has been paid in this respect to the effect exerted on the yields by the following factors, for example: virus strain, size of inoculum, age of embryos at time of inoculation, duration and temperature of incubation subsequent to inoculation. The highly varying behaviour of different strains in the allantoic cavity has already been mentioned. The Enders strain has been found to give better and more constant yields than some other strains (9, 154). Several strains, however, have not been included in the comparison. The impressions of different observers as regards the effect of the size of the inoculum on the yield differ to some extent. Beveridge *et al.* (9) felt that the best yields were obtained with high titre seed. Thus, they routinely used undiluted virus allantoic fluids as inoculum. Seed diluted 10^{-3} usually in their hands gave poor yields of hemagglutinins. Despite their many attempts they were never able to demonstrate »autointerference» phenomena similar to those seen in the case of influenza viruses (75, 76), *i.e.* that the yields with diluted inocula would be better than those obtained with undiluted inocula. According to the observations of Weil *et al.* (172), the yields in allantoic fluids were of the same order when using 10^{-1}, 10^{-2} or 10^{-3} diluted inocula. They considered that the concentration of the virus in the inoculum has no apparent effect on the growth of the virus in the eggs. In the experiments of Green *et al.* (66) the yields of hemagglutinins did not differ significantly with variations in the size of the inoculum from 10 to 300,000 infective doses. Lundbäck, on the other hand, observed that with small inocula the yields of hemagglutinins are less constant than with large inocula (111). In his opinion a seed fluid with a hemagglutination titre of about 10^3 should not be diluted more than 10^{-2} or 10^{-3}. Different investigators agree that 7 to 8 days is a suitable age of chick embryos at the time of inoculation (9, 172,

111, 48). Using 9-day-old chick embryos the yields of hemagglutinins are only slightly poorer than when using 7- to 8-day-old chick embryos (111). In 11-day-old eggs the yields are clearly poorer (111). Weil *et al.* (172) have stated that 7- and 8-day-old embryos give equally good yields if the incubation time is from 4 to 6 days. However, if the eggs are incubated longer than 6 days after inoculation, 7-day-old embryos give greater yields per egg than 8-day-old embryos, because the volume of the allantoic fluid clearly decreases as the age of the embryos exceeds 14 days. Following a small inoculum, the infectivity titres of the allantoic fluids reach a maximum on the 4th to 6th day of incubation (172, 55, 168, 111); the hemagglutination titres rise up to the 6th to 8th day (172, 111). Beveridge *et al.* (9) have expressed the view that +35°C. is the most suitable temperature for incubation of the eggs after inoculation. Higher hemagglutination titres are obtained at +33°C. than at +35°C., but the volumes of the allantoic fluids are smaller at the former temperature. At +36°C., again, the relation between the titres and the fluid volumes is the opposite one.

Irregularities in Propagation. — As already stated, it has proved very difficult or impossible to pass some strains in the allantoic cavity (9, 154, 138, 111). Certain difficulties have even been reported in the cultivation of the strains best adapted to the allantoic cavity. One such difficulty arises from differences in the sensitivities of individual eggs (172, 110, 61, 176, 44, 174). This egg variation has been studied in greater detail by Lundbäck (111). He performed the inoculation under direct visual control so as to be sure that the seed viruses entered the allantoic cavity, and then followed the development of hemagglutinins in individual eggs by means of daily samples of the allantoic fluid. The production of hemagglutinins proved to vary considerably in the different eggs. In some, the hemagglutination titres reached a maximum on the 4th day of incubation, in the other on the 5th, 6th or 7th day, some of the eggs remaining entirely negative during the observation time. The smaller the inoculum, the greater was the proportion of negative eggs. Lundbäck observed that the different batches of eggs differed widely from one another. In the case of some batches, no measurable amount of hemagglutinins formed in the allantoic fluids in one third or more of the eggs, while in other

batches, under similar conditions, hemagglutinins developed in the allantoic fluids of all the eggs. The difference between different batches of eggs was also definitely demonstrated by Gotlieb *et al.* (61). When developing a neutralization test with mumps virus in embryonated eggs, they found the principal difficulty was that the propagation of virus in the allantoic cavity was very inconsistent. Many tests were successful, but at times, usually in the spring and autumn months, the tests tended to fail because a considerable proportion of the eggs did not produce detectable levels of hemagglutinins in the allantoic fluids. When eggs from another poultry farm were used, the tests could again be made as usual. After some time, eggs from the first poultry farm were again used, and were now found to behave in an entirely normal way. It was suspected that some seasonal changes in the composition of the eggs may have been responsible for this problem. No causes related to the care and feeding of the hens have been found to account for the occasional irregular multiplication of mumps virus (9, 61). Even more clearly than in the study mentioned, seasonal variation in mumps virus multiplication in eggs has been demonstrated in the Virus Reference Laboratory of the Central Public Health Laboratory in England (14). For many years, usually from October to February, it has been impossible there to produce antigens in eggs for CF tests owing to poor virus multiplication. Occasionally the virus has multiplied normally in some batches of eggs during such a »bad season», but nevertheless it has proved safer to prepare all the antigens during the favourable seasons.

Analysis of Multiplication. — By comparing the electron microscopic total particle counts of virus preparations of Enders strain with the infectivity titres of the same preparations, Isaacs & Donald (92) arrived at the conclusion that, to initiate infection, the allantoic or amniotic cavity must be inoculated with several hundred virus particles. They assumed the high figure to be due in part to poor adsorption of virus onto the cells (92, 176). In the first few studies concerned with virus propagation in eggs it had already been noted that the production of CF antigen occurs comparatively slowly (67, 104, 7, 8). The opinion regarding the slow reproduction of mumps virus, compared for example with influenza virus, was corroborated by later studies of the production of hemagglutinins and infectivity in allantoic fluids (172, 55).

Not until fairly recently, however, have attempts been made to measure more closely the rate of reproduction. Wolff's results showed that mumps virus is reproduced in about 14 to 17 hours (176). CF antigen could not be demonstrated in the allantoic membranes until 18.5 hours, hemagglutinins not until 19.5 hours after inoculation. The size of the inoculum used in these experiments was not stated. The author says that these results were not always reproducible. Horsfall recently tried to analyse quantitatively the multiplication of mumps virus in the allantoic cavity of chick embryos. He briefly reported his results as follows (88): New virus particles do not appear in the allantoic fluids before 24 hours after inoculation. The constant phase of logarithmic increase lasts for a few days after this, the time required to double the number of the virus particles being 15 hours. If the inoculum is large (initial particle-cell ratio 3 or more) changes occur in reproduction, the most striking being the emergence of hemagglutinating but non-infectious particles.

CULTIVATION IN OTHER HOSTS

Cultivation of mumps virus in the monkey was dealt with above (97, 38, 157). Some old references to successful cultivation in the cat (177) and rabbit (62) have not later been confirmed (157, 40). In recent years successful results have been obtained in many small experimental animals. In the guinea pig, virus multiplication occurs after intraocular inoculation and causes corneal opacity (10). On the other hand, multiplication is more uncertain after inoculation by various other routes (163), but there may be some incomplete growth, as assumed also in the case of the ferret (60). It may be questioned whether multiplication takes place in the lungs of the adult mouse (56); in the lungs of the hamster, multiplication has been observed (21). Mumps virus has also been adapted to the white rat (137). The rapid antibody response of sensitized guinea pigs (152) and hamsters (21) has been suggested as an aid to mumps diagnosis. The virus has been successfully adapted to suckling mice and hamsters (100, 101, 128). It has also been possible to perform neutralization tests in these animals, since the virus is lethal when inoculated intracerebrally (101).

Growth of mumps virus also occurs *in vitro* in different chick embryo tissues, such as the amniotic and allantoic membrane, the brain, and the whole embryo (171, 160, 169, 170, 44, 176). Hemagglutinins have also been observed to form in measurable amounts under these conditions (171). Taylor found that mumps virus causes a cytopathogenic effect in the cells (160), but this finding has not been confirmed by any other authors (170, 171, 176, 44, 169, 13). Using chick embryo tissue cultures the reproduction of mumps virus (175) and the fate of mumps virus in cells (169, 13) have been studied, and a neutralization test has been developed that is claimed to have advantages over the neutralization test in eggs (44). The mumps virus also multiplies in mouse embryo tissue cultures (100).

Egg adapted strains of mumps virus cannot be passed in cultures of the Hela strain of human carcinoma cells and not in cultures of trypsinized monkey kidney cells, but in large doses they have a cytopathogenic effect (72). However, mumps virus strains at early amniotic passage levels in chick embryos multiply in the cells mentioned (73), and these cells evidently are even more sensitive than are embryonated eggs for isolation of mumps virus (73). Certain human transformed cells have been found to sustain persistent, inapparent infections with certain mumps strains (35, 80).

VIRUS - RED CELL INTERACTION

Hemagglutination

Mumps virus has been reported to agglutinate red cells of the following species: man, monkey, horse, cow, bear, pig, sheep, rabbit, guinea pig, and fowl (104, 8, 12, 41, 153, 18, 24, 124). Red cells of the cat (159), the mouse (41, 124) and the ground squirrel (11) have been shown to be unagglutinable by mumps virus. There are reports that some strains are incapable of agglutinating human cells (50, 24, 153). The red cells of individual fowls occasionally give widely varying titres in agglutination tests with mumps virus (8, 110, 135). The effect of the temperature on the hemagglutination titres depends on the cells used (108, 134), the virus strain (108, 24, 153), and the presence of inhibitors (134). Depending upon

these factors the titres may vary greatly as the temperature varies from +4°C. to +37°C.

All accumulated observations indicate that the hemagglutinating property is intimately associated with the mumps virus particle (8, 55, 108, 70, 27, 28). Hemagglutination may thus be used for measuring mumps virus particles. The amount of hemagglutinins is usually measured by Salk's pattern method (143), using fowl cells. According to Isaacs & Donald (92) one agglutinating unit then contains about $10^{7.5}$ virus particles counted by electron microscope. The insensitivity of hemagglutination titration is clearly apparent from this figure. It has been counted that, at partial agglutination end-point, there are about 2 to 3 mumps virus particles per red cell (92).

The adsorption of mumps viruses onto red cells is incomplete, more incomplete than for instance that of influenza A and B viruses (8, 37, 51, 139, 92). Adsorption is poorer in allantoic than in amniotic fluid, but improves if the allantoic fluid is dialyzed against physiological saline (103). At +37°C mumps viruses are eluted from the red cells (4). Some individual viruses, however, evidently are incapable of being eluted, and for this reason red cells treated with mumps virus are agglutinated on adding antiserum (17) and are also able to agglutinate untreated red cells (108).

The enzymatic activity of mumps virus is slight compared with related viruses, so-called myxoviruses (4). In the receptor gradient described by Burnet *et al.* (16) mumps virus is the first, *i.e.*, of all myxoviruses studied, it is least capable of destroying the receptors of other viruses on red cells. The mumps virus also reduces the electrophoretic mobility of the red cells to a less extent than do the other myxoviruses studied (3). If the number of viruses per cell is sufficient and the duration of treatment at +37°C. is long enough, the mumps virus is able to render the fowl cells inagglutinable by other myxoviruses (83).

The hemagglutinin of mumps virus is very sensitive to non-specific inhibitors of hemagglutination (8, 158); the mumps virus is considerably more sensitive for example to the inhibitor of normal allantoic fluid than are influenza virus A and B (8). It has been assumed (22) that mumps viruses cultivated in eggs are heavily contaminated by inhibitors occurring in the tissues and fluids of chick embryos.

Hemolysis

Mumps virus was the first virus shown to possess hemolytic activity (124). Similarly as hemagglutinin, hemolysin is also intimately associated with the virus particle (124, 27, 28). Hemolysin, however, is not identical to hemagglutinin (27, 28, 126). The virus clearly shows stronger hemolytic activity in amniotic than in allantoic fluid, which contains an inhibitor (28, 27, 124). This inhibitor may be calcium (125). The optimal temperature for hemolysis is +37°C and the hemolysis is evidently of enzymatic character (124, 27, 28). A sphingomyelin-like component of the red cell undergoes changes during hemolysis by mumps virus (123).

COMPLEMENT FIXING ANTIGENS

Two complement fixing antigens are produced during mumps virus multiplication (70). One of these, called V antigen (V = virus), is linked to the virus particle itself, the other, called S antigen (S = soluble), is considerably smaller than a virus particle, non-infective, and incapable of being adsorbed onto the red cell. S antigen occurs mainly in infected tissue; there is very little or no S antigen in extraembryonic fluids of chick embryos (70, 41, 103). V antigen is present in the chick embryo both in infected tissues and in the fluids, in greater amount in the latter (70, 41, 103). In addition to V antigen, so-called S′ antigen has been reported to occur in infected amniotic and allantoic fluids; this antigen resembles S antigen but differs from it in its capacity to fix complement under different conditions of testing (103). S′ antigen has also been considered the main antigen in the parotid gland of infected monkeys (103).

Soluble antigens can be separated from V antigen by centrifuging or by the method of adsorption and elution with red cells (70, 103). With both these methods, however, the separation is far from complete. By immunization with purified antigens (103) or by using suitable immunization methods (91) monospecific immune sera, *i.e.* sera reacting only with either soluble antigens or with V antigens, can be prepared. Sera containing both antibodies have also been rendered monospecific by absorption with S or V antigens (70). With such antisera both types of antigen can be measured separately.

During mumps infection in man S antibodies usually are formed more rapidly than V antibodies, but the former also disappear more rapidly (71, 113).

Definite serological differences have never been noted between different mumps virus strains; in hemagglutination inhibition tests, however, some authors have suggested the presence of small variations from one strain to another (9, 138, 110, 175, 24, 155, 161).

PHYSICAL AND CHEMICAL PROPERTIES

Structure

The size of mumps virus has been measured by filtration (67, 37, 42), by analytical centrifugation (37), and by electron microscopic methods (37, 172, 33, 139). The results indicate a wide variation in particle size. The average values are of the order of 100 to 200 mμ. On the basis of electron microscopic studies, the shape of the mumps virus has been described as follows: Dome shaped body (37), relatively flat (172, 37, 33) — especially the largest forms (37), roughened, irregular, indefinite outline (172, 139). In formalinized preparations the particles are smaller, more spherical and more uniform (172, 139). The virus particles of different strains have not been found to differ in size and appearance (139). Filamentous forms of virus have not appeared in mumps virus preparations (33, 139). On electron microscopic observations, mumps virus has been assumed to consist of a ring of ribonucleoprotein enclosed within a protein coat (166).

Stability

It has been observed that at about —70°C. the infectivity of several mumps virus strains in amniotic and allantoic fluids is preserved for several months without any noticeable decrease (55, 42, 101, 61, 44). The infectivity is also fairly well preserved at —20°C (127). Observations regarding the stability of infectivity at +4°C have varied widely. According to Weil *et al.* (172), the Enders strain was very unstable under any of the conditions studied. In two weeks the infectivity of an allantoic fluid preparation decreased 1.6 log at +4°C. Under similar conditions, however,

Penttinen (136) found the infectivity of the Enders strain was preserved for over 3 months at least, without any significant decrease. The Enders strain has been reported to lose its infectivity at room temperature within 4 days (42). Penttinen (136) observed that an Enders strain preparation kept in dark at room temperature in the presence of 20 per cent human albumin, retained some infectivity for more than 6 months. Kilham (102) showed that some recently isolated strains retained infectivity after being stored for 3 months at room temperature. Horsfall reported that at + 35°C the half-life of mumps virus in allantoic fluid was 80 minutes (88).

The infectivity of mumps virus is readily inactivated by formalin, ether and ultraviolet irradiation (67). It is possible by means of ultraviolet irradiation to destroy the infectivity of mumps virus with preservation of other properties, such as interfering activity (78, 162), hemolysin (27), hemagglutinin (78, 27, 162), and complement fixing activity (67). The different properties of mumps virus are destroyed by heat in the order indicated: infectivity — hemolysin — hemagglutinin — V antigen — S antigen (67, 104, 8, 108, 27, 28, 103). At +4°C the hemagglutinating and complement fixing activity are very stable, being preserved almost unchanged for several months (9, 108, 5). Subfreezing temperatures are considered definitely more deleterious to the complement fixing activity of mumps virus (5). Differences have been observed in the stability of hemagglutinins of different mumps strains at room temperature and higher temperatures (23, 54, 102).

»INCOMPLETE» VIRUSES

INFLUENZA VIRUS

von Magnus Phenomenon

Henle & Henle (75, 76) dealt with the drawbacks that may be associated with the use of undiluted inocula when passing influenza virus in embryonated eggs. A large proportion of the viruses may be inactivated *in ovo* during prolonged incubation or *in vitro* during storage, losing their infectivity but retaining their interfering ability. If seed virus of this kind is inoculated in undiluted state,

the yields may be very poor or the whole virus strain may be lost, since the inactivated viruses interfere with the multiplication of the active viruses present. This »autointerference» can be eliminated by diluting the seed virus. Thus, for good yields to be obtained, diluted, small inocula are most reliable.

Later, in analysing the propagation of influenza virus in chick embryos, von Magnus (115—119) directed attention to a different kind of phenomenon which became apparent when undiluted inocula were used during serial passages. As usual, high hemagglutinin titres developed in the allantoic membranes and fluids, but the infectivity titres were considerably lower than ordinarily observed after small inocula. This discrepancy of the results obtained with these two methods of virus titration increased further when several successive passages were made with undiluted inocula. The ratio between the infectivity and the hemagglutination titres could decrease as much as to about 10^2, whereas with standard virus preparations (obtained with very small inocula) it was regularly about 10^6. From this von Magnus inferred that during serial passages with undiluted inocula the great majority of the viruses produced in the allantoic membranes and fluids were hemagglutinating but noninfectious. These were termed by von Magnus incomplete viruses.

The term 'von Magnus phenomenon' has been applied to these events occurring during serial undiluted allantoic passages of influenza virus. This phenomenon has been highly reproducible (54, 36, 120, 46, 19, 49, 20, 34, 121).

Production of Incomplete Virus

Different influenza virus strains differ as regards their tendency to produce incomplete viruses (45, 46, 120). The production of incomplete viruses evidently requires adsorption of several virus particles per host cell (93) within a narrow period of time (109). In addition to the size of the inoculum, its composition is of essential importance. After inoculation of standard virus, though the inoculum might be very large, the yield of incomplete viruses is considerably less than after a large inoculum containing incomplete (120, 79) or heat inactivated viruses (86, 120, 87, 49, 129, 130) in addition to standard viruses. It has been stated that, even with

a small inoculum, large amounts of incomplete viruses form in the allantoic fluids if the cells of the allantoic membranes are treated with periodate (47). This opinion was later challenged (109). However, even after small inocula of standard viruses, noninfectious hemagglutinins have been found in the allantoic membranes, but these differ from incomplete viruses (64, 79). The occurrence of the von Magnus phenomenon has been claimed to depend upon the temperature of incubation: at +33°C this phenomenon was not detectable (34).

After a large inoculum, incomplete viruses have been demonstrated not only in the allantoic membrane and fluid of the chick embryo but also in other host-virus systems, for example in deembryonated eggs (6, 19), in tissue cultures (32, 74, 65), in mouse brain using non-neurotropic virus strains (145), and in mouse lung using virus strains capable of causing lesions (57, 58). In these host-virus systems incomplete viruses are already produced in very large amount in the first undiluted passage.

Properties and Nature of Incomplete Virus

Incomplete virus is capable of immunizing (116, 120). Incomplete and standard viruses evidently resemble one another as regards enzymatic (156) and hemagglutinating activity (173, 36). In electron micrographs, incomplete viruses are larger, flatter and more heterogeneous than infectious viruses (173, 167), they are less dense (167, 84), and have little or no internal structure detail (132). Compared with standard viruses, incomplete viruses have a lower sedimentation constant (52, 68, 53), a lower ribonucleic acid content (1, 2), a higher lipid content (165), and less internal S antigen in the virus particles (107). The toxicity of incomplete viruses for mice is less than that of standard viruses (77, 6, 98, 114, 121). Incomplete viruses are capable of forming a genetic recombinant with standard viruses (19). After ultraviolet irradiation the interfering activity of incomplete viruses is equally good as that of standard viruses; however, the interfering activity of incomplete viruses weakens when their internal S antigen drops below a certain limit (131). At least some of the incomplete viruses seem capable of producing noninfectious hemagglutinins, even though they cannot multiply continually after a small inoculum (19). Thus,

there may be differences in degree in the reproducibility of incomplete viruses. Incompleteness seems to differ in degree also with regard to many other properties of influenza virus (68, 53, 120, 165, 173, 1, 49, 2, 107, 132).

The origin and nature of incomplete viruses have been much discussed. Most authors regard the opinion, first presented by von Magnus (118), that incomplete virus represents an intermediate stage in the normal reproduction of influenza virus as most probably correct (6, 112, 54, 89, 79). However, the possibility of incomplete viruses being a product of an abnormal virus-cell interaction has also been considered (146). Some authors have advanced the opinion that incomplete viruses might be a degradation product of standard viruses (86, 87, 34), but biological (120, 79), physical (53, 84, 132), and chemical (165, 1, 2) differences between incomplete viruses and heat inactivated viruses point against this opinion.

OTHER VIRUSES

Newcastle Disease Virus (NDV)

Von Magnus phenomenon has not been demonstrated with NDV (64, 88). Even after small inocula, noninfectious hemagglutinins have been demonstrated, in addition to infective virus particles, in the allantoic membranes of chick embryos but scarcely in the allantoic fluids (64). These noninfectious hemagglutinins are nonhemolytic and their size and density evidently differ considerably from the corresponding properties of infectious viruses, because their sedimentation is much poorer (63, 64). The hemagglutinins concerned have been assumed to be precursors of fully infectious viruses (64).

Fowl Plaque Virus

Hemagglutinating, noninfectious, »incomplete» viruses have been found in the allantoic membranes of chick embryos infected with fowl plaque virus. These »incomplete» viruses are larger, flatter and more heterogeneous than are active viruses, they contain less desoxyribonucleic acid, and they are more slowly sedimented by ultracentrifugation than active viruses (147, 148, 149).

Vesicular Stomatitis Virus (VSV)

The yields of infectious virus decrease considerably after successive undiluted passages of VSV in tissue cultures of chick embryo cells. Poor yields are also obtained when using as inocula mixtures of infectious viruses and virus material from undiluted passages. The yields improve if the inoculum is diluted. These phenomena have been accounted for by the fact that an interfering component called T is produced in serial undiluted passages. The best yields of T are obtained with large inocula containing both infectious viruses and T. T has been assumed to be an incomplete form of vesicular stomatitis virus (30).

Rift Valley Fever Virus

The yields of infective virus vary very greatly during serial undiluted passages of RVF virus in mice. This fluctuation of the titres shows a distinct regularity. The virus preparations from the passages preceding the fall in the titres contain interfering material which is evidently noninfectious but capable of immunizing. This material has been considered analogous to incomplete forms of influenza virus (122).

Miscellaneous Viruses

The noninfectious particles found during reproduction of certain bacteriophages and plant viruses have also been assumed to be analogous to incomplete forms of influenza virus (120).

CONCLUSIONS FROM THE LITERATURE AND SCOPE OF THE PRESENT INVESTIGATION

Large amounts of virus material referred to as »incomplete» viruses emerge during the multiplication of some viruses under certain conditions. Many data are compatible with the hypothesis that »incomplete» forms of this kind are virus »precursors» or »building stones» arising through virus-cell interaction. However, in the case of no virus has the relationship of such »incomplete» forms to normal virus multiplication been definitely clarified. Conclusive evidence is also lacking as to whether the »incomplete» forms of different viruses are truly analogous. Although at present the nature of »incomplete» viruses is not clear, analysis of their origin and properties may well provide a clue to a better knowledge of virus-cell interaction.

One method by which »incomplete» forms of several viruses have been produced in abundance is to carry out several successive passages with undiluted inocula (118, 30, 122). — To the present author's knowledge, *serial* undiluted passages of mumps virus in the allantoic cavity of chick embryos have not earlier been analysed from this point of view.

The inconsistency of mumps virus multiplication in the allantoic cavity of embryonated eggs is a phenomenon familiar to many investigators (9, 110, 154, 61, 155, 111, 136, 14). In trying to find optimal conditions assuring the best yields, attention has been directed also to the size of the inoculum. In none of these studies, however, have inocula of varying size been systematically compared as to their effect on the yields *during several successive passages.*

From this basis the present author has approached the subject of mumps virus multiplication in the allantoic cavity of chick

embryos during successive passages with undiluted (UP-series) and highly diluted (ST-series) inocula. Especially the following points will receive attention in the experiments to be presented:

Yield of hemagglutinins in various UP- and ST-series

Hemagglutination-inhibiting capacity of allantoic fluid preparations from UP-series

Relationships of infectivity, hemagglutination, and complement fixation titres in allantoic fluid preparations during UP- and ST-series

Interfering activity of allantoic fluid preparations from UP-series and of inactivated mumps virus

PRESENT INVESTIGATION

TERMS AND ABBREVIATIONS USED

Undiluted passage (UP) = passage carried out with undiluted inoculum

UP-series = successive undiluted passages

UP-viruses = viruses obtained by undiluted passages

Standard passage (ST) = passage carried out with 10^{-4}—10^{-6} diluted inoculum; incubation time 5 days

ST-series = successive standard passages

ST-viruses = viruses obtained by standard passage(s)

ST-seed = inoculum consisting of ST-viruses

Δ ST-viruses = standard viruses inactivated at +35°C *in vitro*

MATERIAL AND METHODS

Viruses

Enders strain, adapted to the allantoic cavity, was supplied in 1949 by Dr. Herbert R. Morgan. At the State Serum Institute in Helsinki it has since been carried through 59 allantoic passages. In the experiments to be presented the strain ranged from the 60th to the 149th passage.

Habel strain was obtained from Dr. Victor J. Cabasso in 1955 in the 23rd egg passage. After four amniotic passages the strain was freeze-dried. In January 1957 cultivation of the strain was resumed, starting with a freeze-dried ampule. After eight more amniotic passages the first attempt was made to pass the virus allantoically. The yield of hemagglutinins was irregular in the first few passages but after about ten passages the allantoic fluids began to show hemagglutinins regularly. In the experiments to be reported the strain ranged between the tenth and the 94th allantoic passage.

KS strain was isolated by the present author from the saliva of a mumps patient in January 1957. Not until the third amniotic passage was hemagglutination positive in the amniotic fluid. After eight amniotic passages the first attempt was made to pass the strain in the allantoic cavity, and since the first allantoic passage the strain has grown well in the allantoic cavity. The virus was identified as mumps virus by the hemagglutination inhibition test with anti-Enders fowl serum and by the complement fixation test with a known positive human convalescent serum. In the experiments to be reported the strain ranged between the second and the 40th allantoic passage.

HeWe strain was isolated by the author from the saliva of a mumps patient in February 1957. The amniotic fluid in the first passage already gave a positive hemagglutination. After four amniotic passages the first attempt was made to pass the strain allantoically, and the virus has grown well in the allantoic cavity since the first passage. The virus was identified as mumps virus in the same way as the KS strain. No differences could be demonstrated between the four strains in cross hemagglutination inhibition tests using fowl immune sera prepared against all the four mumps virus strains mentioned. In the experiments to be presented the HeWe strain ranged between the first and the 39th allantoic passage.

During the last experiments in this study it was checked that the anti-Enders strain fowl serum still inhibited hemagglutination by all the strains used.

Eggs

Eggs of White Leghorn hens were used in all experiments. The eggs for the majority of tests were obtained from one and the same poultry farm. In the later stages of this study it was no longer possible to obtain eggs from this farm, and therefore eggs from two other poultry farms had to be used. The infectivity titres of two virus preparations were deter-

mined in parallel using the eggs from these farms, and no differences were noted. The eggs used in each individual test series, however, were always from one and the same farm.

Before inoculation the eggs were incubated for 7 to 9 days at +37 to 38°C.

Allantoic Passages

Preparation of Inoculum. — Sterile tryptose phosphate broth was used as virus diluent. To all inocula were added 500 units of G-penicillin and 500 γ of dihydrostreptomycin per ml. Except where otherwise indicated, the volume of the inoculum was 0.2 ml. In some experiments the volume of the inoculum was 0.5 or 1.0 ml. and this is indicated separately.

Inoculation Technique. — Eggs incubated for 8 days, more rarely 7 or 9 days, were used in the allantoic passages. The air sac was localized by candling, and the point of inoculation was marked at an avascular site exactly on the boundary of the amniotic and the allantoic sac. (The contours of the amniotic sac could be clearly seen when candling the end opposite to the air sac.) The located areas were wiped with alcohol. The shell was pierced with an awl at the site corresponding to the air sac, and a small hollow was very carefully made at the point of inoculation marked on the shell. The eggs were placed at an angle of 45 degrees, the site of inoculation and the air sac upwards. At the site of inoculation the shell was pierced with a 16 gauge needle almost parallel to the surface of the egg, and the inoculum was injected to a depth of about 3 mm. The holes were stopped with liquid paraffin. The egg was turned in vertical position with the air sac upward and incubated at +35°C for 2, 3 or 5 days as noted separately in the case of each experiment.

Harvesting of Allantoic Fluids. — In some experiments, the eggs had been chilled overnight at +4°C before harvesting; in most later experiments, however, the fluids were harvested without previous chilling of the eggs. It is indicated in the text which of these ways was used in each experiment. The shell above the air sac was wiped with alcohol and then removed with sterilized forceps. The allantoic fluid was harvested with a sterile syringe. The shell membrane was not removed because its removal could cause admixture of blood in the allantoic fluid; the needle was pushed direct through the shell membrane into the allantoic cavity and the fluid was aspirated into the syringe.

Pooling of Allantoic Fluids. — If allantoic fluids from different eggs were pooled, care was taken as far as possible to harvest an equal amount of each fluid.

Storing of Virus Preparations

As far as possible all passages and infectivity titrations were performed immediately without storing the virus fluids. If some virus preparations had to be stored, they were kept at +4°C, except in a few test series, separately noted later, in which virus fluids were stored at —20°C. Storing

at —20°C was avoided, since it has been observed that subfreezing temperatures may be more deleterious than +4°C to the complement fixing antigens and hemagglutinins (133, 5).

Red Cells

The red cells of different fowls may give considerably diverging results in hemagglutination titrations with mumps virus (8, 110, 135). This was eliminated as far as possible in the present study by titrating several mumps virus preparations parallelly with red cells of several hens and roosters and by choosing as blood donors for all later experiments only those fowls whose cells gave the highest and identical titres.

The blood was drawn from the wing vein into a syringe containing 3.8 per cent sodium citrate solution. The red cells were washed three times with sterile saline and were stored under sterile conditions in 10 per cent solution at +4°C. In some of the agglutination titrations performed, the sensitivity of the cells did not seem to change within one week of storage. Older cells were discarded.

Hemagglutination Titrations

Serial two-fold dilutions of the virus preparation in volumes of 0.5 ml were prepared on a plastic plate (144). Saline (pH buffered to 7.5 by adding 1/10th part 1/15 M Sørensen phosphate buffer) served as diluent. In most titrations pipettes were changed with each hole. In the case of some large titration series, in which the hemagglutination titres of individual eggs were determined, pipettes were only changed with every third hole. Some titrations were performed in duplicate. 0.5 ml of 0.5 per cent fowl red cell suspension was added to each hole. The cells were mixed by shaking the plate. The plates were kept at room temperature (18—22° C) and the results read after one hour. A dilution giving partial agglutination (±) was taken as the end-point. If agglutination was complete (+) in the last positive hole, the geometrical mean corresponding to the dilutions at this hole and the next one was considered the end-point. In the present study, then, one hemagglutinating unit (HA) indicates the smallest amount of virus capable of partially agglutinating, in the pattern test as described above, 0.5 ml of 0.5 per cent chicken erythrocyte suspension, *i.e.* about $10^{7.15}$ red cells (44 a, 36). All the hemagglutination titres are expressed in this study as logarithms of hemagglutinating units per ml of allantoic fluid (HA/ml) so as to render possible a comparison between hemagglutination and infectivity titres.

Reproducibility of Hemagglutination Titrations. — The reproducibility of the hemagglutination titrations was investigated by titrating the same virus allantoic fluid several times. Twenty-three dilution series were made, changing pipettes with each hole, and 31 dilution series, changing pipettes with every third hole. In the former titration series, the mean of the titres

was 2.86 ± 0.02 and in the latter 3.02 ± 0.02. When the dilution series were made changing pipettes with every third hole, the titres obtained were thus on an average 0.16±0.03 log higher than when changing pipettes with each hole. The standard deviation (31) of a hemagglutination titre, calculated on the basis of the results, is about 0.1 regardless of whether pipettes had been changed with each hole or with every third hole. Thus, a difference of 0.35 log or more between two individual titres is significant with 95 per cent probability if the dilution series in both cases are made in the same way.

Infectivity Titrations

A ten-fold dilution series of the virus allantoic fluid was prepared in sterile tryptose phosphate broth in Kahn tubes. Individual pipettes were used with each tube. Into each tube were added 500 units of G-penicillin and 500 γ of dihydrostreptomycin per ml. Inoculation was carried out immediately after this. Five or more dilutions estimated to lie nearest to the end-point were adopted for inoculation; each of these dilutions was inoculated allantoically into at least 6 eggs, in some titrations into 8 to 10 eggs. The volume of the inoculum was always 0.2 ml. The eggs had been incubated for 8 to 9 days before inoculation. After inoculation the eggs were incubated for 6 days at +35°C. The allantoic fluid was harvested from each egg individually. The techniques of inoculation and harvesting have been described above. The allantoic fluid was examined for the presence of hemagglutinins by adding 0.5 ml of 0.5 per cent fowl erythrocyte suspension into the Kahn tubes containing 0.5 ml of undiluted allantoic fluid. The tubes were shaken, kept at room temperature, and the results read after one hour. The 50 per cent infectivity end-point was calculated by the method devised by Reed & Muench (140) and the results were expressed as the number of infective doses per ml of allantoic fluid studied (EID_{50}/ml). The infectivity titres are expressed in this study in logarithmic units.

When determining the presence of hemagglutinins the allantoic fluid was not diluted, because the hemagglutinin titres were found to be very low in some eggs. In normal allantoic fluids derived from 14- to 15-day-old embryos, the cells were never agglutinated to a disturbing extent in agreement with earlier observations (29). In one experiment it was also observed that, when inoculating 8-day-old embryos with 0.2 ml of tryptose phosphate broth containing antibiotics (see above), no fowl cell agglutinins formed during 6 days' incubation in the allantoic fluids of 42 eggs.

Reliability of Infectivity Titrations. — The reproducibility of infectivity titrations was studied by making 16 parallel titrations of one and the same virus preparation. All 16 dilution series were separately prepared. Each dilution was inoculated into 6 nine-day-old eggs. The results obtained are given in table 1. The standard deviation of the infectivity titres (31) is 0.3. Accordingly a difference of over 0.9 log between two individual infectivity titres should be significant with 95 per cent probability (31).

TABLE 1

RESULTS IN SIXTEEN PARALLEL INFECTIVITY TITRATIONS OF ONE MUMPS VIRUS PREPARATION

Individual Titrations	Dilution of Inoculum 10-7	10-8	10-9	10-10	50 % End-point[1] (log)	EID_{50}/ml[2] (log)
1	6/6 [3]	3/5	0/6	0/6	8.2	8.9
2	5/6	3/6	2/5	1/6	8.3	9.0
3	6/6	5/6	1/6	0/6	8.5	9.2
4	4/4	5/5	0/6	0/5	8.5	9.2
5	6/6	5/6	1/6	0/4	8.5	9.2
6	5/6	4/5	1/5	1/6	8.5	9.2
7	5/5	6/6	0/6	0/6	8.5	9.2
8	4/5	4/6	3/5	0/6	8.6	9.3
9	6/6	6/6	1/5	0/6	8.6	9.3
10	4/6	6/6	2/6	1/6	8.7	9.4
11	5/6	4/5	3/6	0/6	8.7	9.4
12	6/6	4/6	3/6	1/6	8.8	9.5
13	5/6	4/5	3/6	1/6	8.8	9.5
14	6/6	6/6	2/6	1/5	8.9	9.6
15	5/6	4/5	4/6	2/6	9.2	9.9
16	6/6	6/6	4/6	0/5	9.3	10.0

[1] Computed after Reed & Muench (140).

[2] Egg infective doses per ml of preparation studied.

[3] Numerator = number of eggs containing detectable levels of hemagglutinins.
Denominator = total number of eggs inoculated.

— Each infectivity titre is calculated according to the distribution of positive and negative eggs in one group of infected eggs (140). It has been observed that the sensitivity of individual eggs and of different batches of eggs, even from one and the same poultry farm, to mumps virus inoculated into the allantoic cavity can vary within wide limits (61 111, 14). These phenomena also appeared from the present study (page 50). Because of this, the author feels that, when comparing two individual titres obtained in titrations *at different times*, the »confidence limit» of 0.9 log is not any longer very reliable.

Complement Fixation Titrations

The titrations were performed in Kahn tubes.

Saline was used as diluent.

The *hemolytic system* consisted of equal parts of 2.5 per cent sheep cells and the appropriate dilution of hemolysin containing two full units. Sensitizing was allowed to take place at room temperature for 15 minutes.

The *complement* used was pooled guinea pig serum which had been

stored for at most one week at —20°C. The complement was titrated in the presence of each of the antigens used. 1.26 full units of complement were used in the test proper and 1.26, 1 and 1/1.26 full units in the complement controls.

Immune Serum. — One and the same human mumps convalescent serum was used in all experiments. It had been taken 3 weeks after the onset of the disease, and the titre was 1/128 using optimal dilution of crude mumps allantoic fluid antigen. The serum was stored at —20°C without inactivating and without preservatives. Before each test the required quantity of serum was inactivated for 30 minutes at +56°C. The serum dilution in all experiments was the same, 1/8, *i.e.* 16 antibody units.

The Test Proper. — Unit volumes of 0.2 ml were used throughout the test, the final volume in each tube being 1.0 ml.

A two-fold dilution series of the allantoic fluid preparation under study was made, starting from 1/1. To each tube was added serum diluted 1/8 and complement in suitable dilution. The tubes were incubated at +37°C for 45 minutes or, in some experiments, allowed to stand overnight at +4°C. After this, 0.4 ml of sensitized cells was added to each tube, and the tubes were incubated at +37°C until the serum control and the antigen control were completely hemolyzed. The tubes were then at once placed at +4°C, and the results were read the next morning. The last dilution of the allantoic fluid preparation studied showing 50 per cent fixation (±) was considered the titre of the allantoic fluid. If there was complete fixation (+), *i.e.* no hemolysis, in the last positive tube, the geometrical mean of the dilutions corresponding to this tube and the next one was taken as the titre. All titres are expressed as logarithms of reciprocals.

No essential differences in the titres were noted regardless of whether the short (45 min. at +37°C) or the long fixation time (over night at +4°C) was used.

Controls. — The controls used in each test series are presented in table 2.

TABLE 2

CONTROLS IN COMPLEMENT FIXATION TESTS

Control tube No.	Complement			Unknown Antigen	Control Positive Antigen	Normal Allantoic Fluid	Immune Serum	Hemolytic System
	1.26× unit	1 × unit	1/1.26 × unit					
1	×							×
2		×						×
3			×					×
4	×			×				
5	×				×		×	×
6	×					×	×	×
7	×						×	×

YIELD OF HEMAGGLUTININS AND COMPLEMENT FIXING ANTIGEN(S) DURING SERIAL UNDILUTED PASSAGES (UP-SERIES) AND STANDARD PASSAGES (ST-SERIES)

Yield of Hemagglutinins and Complement Fixing Antigen(s) during UP- and ST-series with Incubation Time of Five Days in Each Passage

To compare the yields of hemagglutinins in undiluted passages and in standard passages of Enders strain, five passage series were made simultaneously; in three of these series the transfers were always performed with an undiluted inoculum, in two series the inoculum was always diluted 10^{-4}. In all five series an average of 6 eggs were always inoculated, and the hemagglutination titration and transfer were performed with pooled allantoic fluids. A total of 64 undiluted passages and 32 standard passages were thus carried out. The hemagglutination titres in these passages are presented in figure 1. It shows that the hemagglutinin titres of the allantoic fluids were invariably high in the standard passages. In undiluted passages, however, the situation is markedly different: in some passages the titres were high, about equally high as in

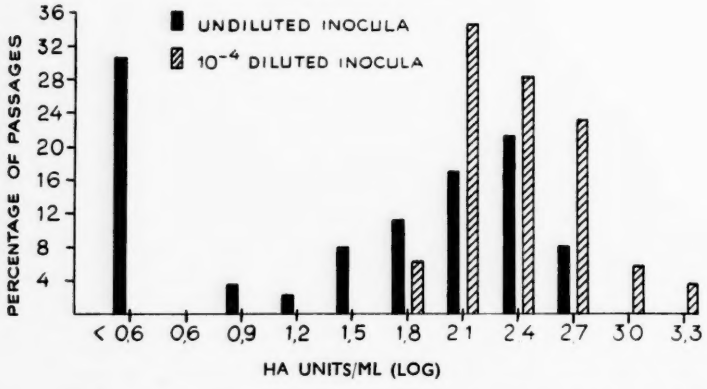

Fig. 1. — *Enders strain.* Dispersion of hemagglutinin titres of pooled allantoic fluids (average 6 eggs) in 64 undiluted *) and in 32 standard **) passages.

*) Three parallel series (A, B and C) consisting of 22, 21 and 21 successive passages respectively.

**) Two parallel series (A and B) consisting of 22 and 10 successive passages respectively.

standard passages, but in about one third of the passages measurable amounts of hemagglutinins did not form in the allantoic fluids.

A similar study was carried out with the Habel strain. The dispersion of the hemagglutination titres of a total of 62 undiluted passages and 32 standard passages is shown in figure 2.

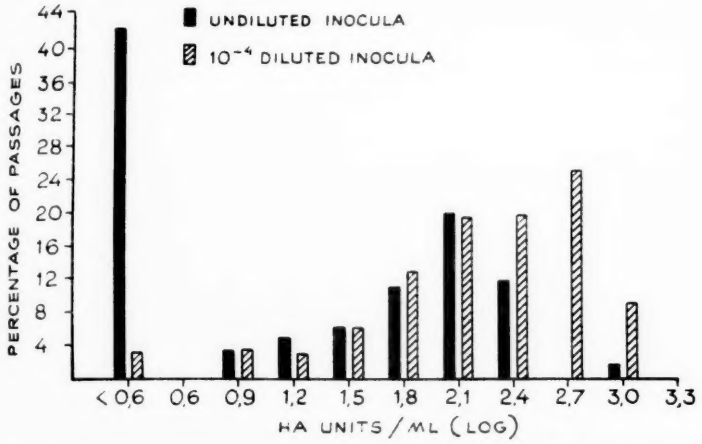

Fig. 2. — *Habel strain.* Dispersion of hemagglutinin titres of pooled allantoic fluids (average 6 eggs) in 62 undiluted *) and in 32 standard **) passages.

*) Three parallel series (A, B and C) consisting of 22, 20 and 20 successive passages respectively.

**) Two parallel series (A and B) consisting of 22 and 10 successive passages respectively.

A phenomenon similar to that occurring with the Enders strain is seen. However, compared with the Enders strain, the hemagglutinin titres were lower and less constant. In some standard passages the hemagglutinin titres were very low, in two passages (about 3 per cent) even below 0.6 log. In undiluted passages, however, the hemagglutinin titre was below 0.6 log in 42 per cent of all passages, *i.e.* in almost every second passage detectable amounts of hemagglutinins were not produced in the allantoic fluids.

In both the test series reported, especially the latter (Habel strain), hemagglutination could be negative in several successive undiluted passages. However, the virus strain was never lost during the undiluted passages: even after several negative passages hemagglutinins appeared again in the allantoic fluids.

During these two experimental series the titres of both strains

showed a definite rising tendency in the standard passages. Thus the titres obtained in the later passages tended to be higher than those obtained in the early passages.

In all the above test series, the virus allantoic fluids had been stored between passages for periods of varying length at —20°C or +4°C.! In the experiments to be reported below, however, fluids were never stored between passages: transfers were always made immediately.

Figure 3 shows the hemagglutinin titres in parallel UP- and ST-series of Enders strain. Each hemagglutinin titre represents a pooled allantoic fluid from 6 eggs on an average. The maximal variations in the hemagglutinin titres in the standard passages were

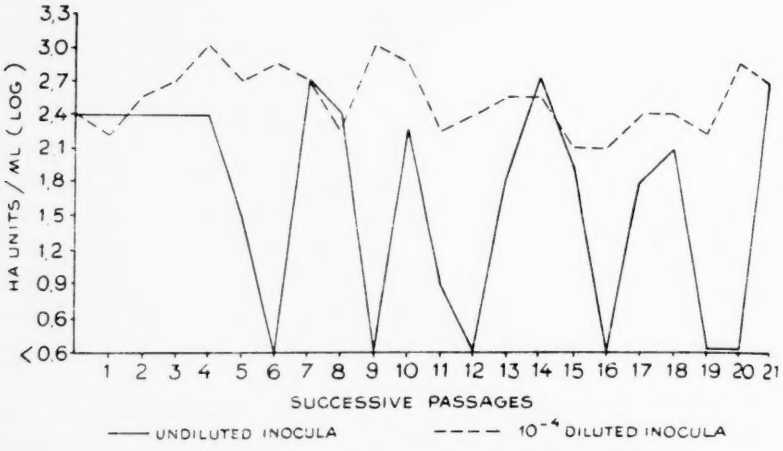

Fig. 3. — *Enders strain*. Hemagglutinin titres of pooled allantoic fluids (average 6 eggs) in UP- and ST-series with incubation time of 5 days in each passage.

0.9 log, *i.e.* eight-fold. In the first four successive undiluted passages the titres obtained in the allantoic fluids were high and exactly similar, but in the subsequent passages the hemagglutinin titres dropped abruptly. No hemagglutinins whatever were detectable in the pooled allantoic fluid from the sixth undiluted passage. In the seventh passage the titre rose to the initial level, to fall again markedly in the ninth passage. During the subsequent passages such a fluctuation of the hemagglutinin titres occurred over and over again. The maximal titre fluctuations in different undiluted passages were over 2.1 log, *i.e.* over 128-fold.

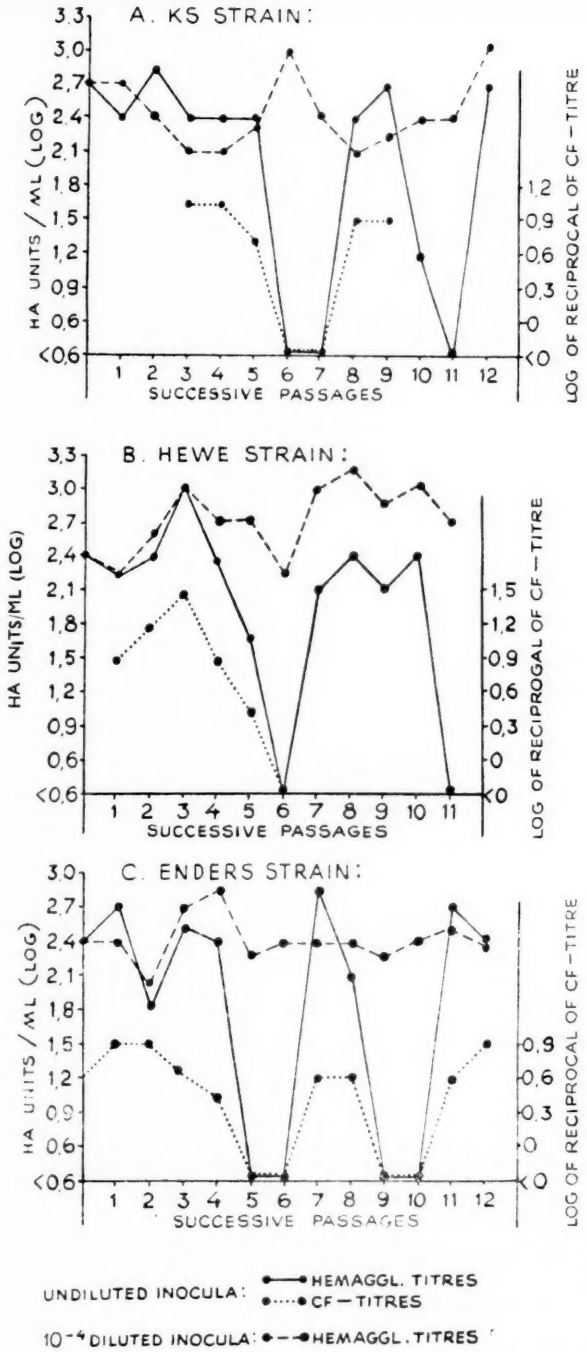
A. KS STRAIN:
HA UNITS / ML (LOG)
LOG OF RECIPROCAL OF CF – TITRE
SUCCESSIVE PASSAGES
B. HEWE STRAIN:
HA UNITS/ML (LOG)
LOG OF RECIPROGAL OF CF – TITRE
SUCCESSIVE PASSAGES
C. ENDERS STRAIN:
HA UNITS / ML (LOG)
LOG OF RECIPROCAL OF CF – TITRE
SUCCESSIVE PASSAGES
UNDILUTED INOCULA: HEMAGGL. TITRES
CF – TITRES
10^{-4} DILUTED INOCULA: HEMAGGL. TITRES

Fig. 1. — (A, B, C.) Hemagglutination and complement fixation titres of pooled allantoic fluids (average 6 eggs) in UP- and ST-series. Incubation time in each passage 5 days.

In figure 4 it is seen that this kind of fluctuation in hemagglutinin titres during successive undiluted passages also occurred in the case of two other mumps virus strains. The titres dropped below 0.6 log in some passages. The maximum titre fluctuations in different passages were over 2.25 log, *i.e.* over 180-fold, with the KS strain and over 2.4 log, *i.e.* over 256-fold, with the HeWe strain. In the standard passages, however, the maximum fluctuations of titres were at most 10-fold.

Figure 4 in addition gives the antigen titres of the UP-allantoic fluids in complement fixation tests. The allantoic fluids of each virus strain were titrated in one and the same experiment. It is seen that, with all the strains used, the complement fixing capacity of the allantoic fluids from UP-series decreased with their hemagglutinating capacity. The allantoic fluids having hemagglutinin titres below 0.6 log also never contained detectable amounts of CF antigen(s).

In order to compare different mumps virus strains as regards variations in their hemagglutinin titres during undiluted passages, UP-series of four different strains were carried out concurrently, taking care as far as possible to make the conditions for all the strains identical.

All four virus strains had been passed through at least ten standard passages, in the last of which the allantoic fluid was harvested without previous chilling of the eggs. Immediately after hemagglutination titration, about $10^{2.7}$ agglutinating units of each strain were inoculated into the allantoic cavity of 10 eggs. The size of the inoculum was always 1.0 ml in these experimental series. The fluids were always harvested without previous chilling of the eggs and were easily obtained without admixture of blood. In each passage, 10 eggs were always inoculated with each virus strain. The embryos survived well in spite of the large inocula: not more than 1—2 in each group died, usually none.

The results are given in figure 5, which shows the fall in the titres to occur in the case of all strains between the fourth and the sixth passage. The results are in harmony with many other experiments in which the decrease in the titres of Enders and Habel strains in UP-series were compared: the titres of the Habel strain always dropped earlier than those of the Enders strain, similarly as in the experiment presented in figure 5.

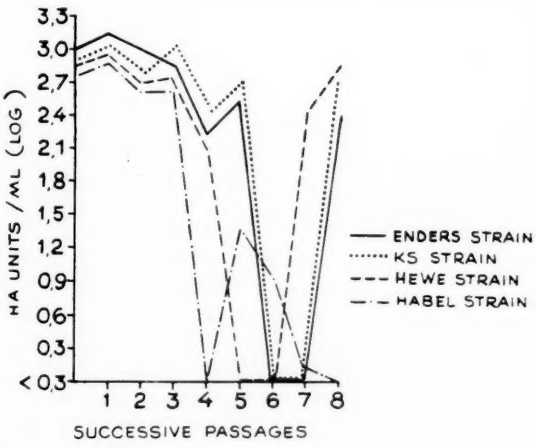

Fig. 5. — Hemagglutinin titres of pooled allantoic fluids (8—10 eggs) in parallel UP-series of four mumps strains. Incubation time in each passage *5 days*.

The total number of those UP-series of Enders strain in which the incubation time in each passage was 5 days and transfers were made immediately without storing the fluids between passages, was 7. The hemagglutination titres in these UP-series dropped as follows: In the fifth passage in one series, in the sixth passage in five series, in the seventh passage in one series, and in the ninth passage in one series (fig. 14, page 54).

Altogether five corresponding UP-series with the Habel strain were performed. The fall in hemagglutination titres occurred in the third passage in two series, in the fourth in three series.

Yield of Hemagglutinins during UP-series with Incubation Time of Two and Three Days in Each Passage

The following experiments with Enders and Habel strains aimed at establishing whether a fluctuation of hemagglutination titres as described above occurs in undiluted passages if the incubation time is shorter than 5 days. Two UP-series were carried out with each strain; in one the incubation time was 2 days, in the other 3 days. Those UP-series of both strains in which the incubation time was equally long were performed parallelly.

In each passage both virus strains were inoculated into 10 embryos. The volume of the inoculum was 0.5 ml. Harvesting of the allantoic fluids was always made without previous chilling of the eggs. The fluids were not stored between passages: the transfers were always made with pooled allantoic fluid. Each hemagglutination titre represents pooled allantoic fluids from 8 to 10 eggs.

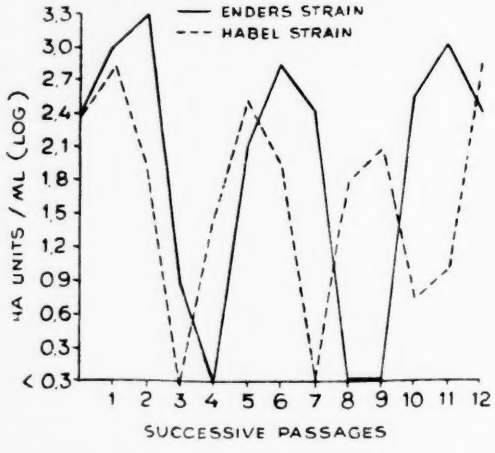

Fig. 6. — Hemagglutinin titres of pooled allantoic fluids (average 10 eggs) in parallel UP-series of Enders and Habel strains. Incubation time in each passage *3 days*.

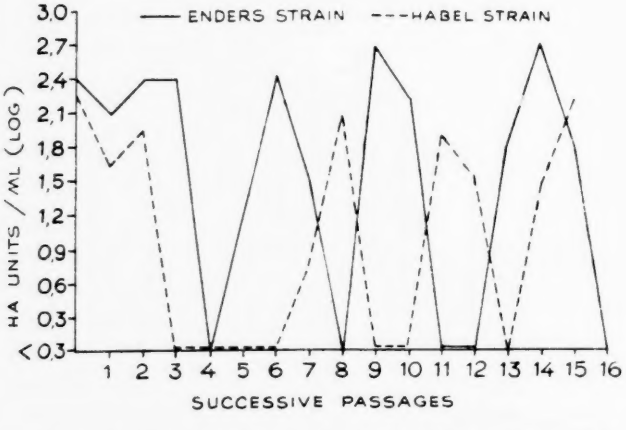

Fig. 7. — Hemagglutinin titres of pooled allantoic fluids (average 10 eggs) in parallel UP-series of Enders and Habel strains. Incubation time in each passage *2 days*.

In the UP-series with 2 and 3 days' incubation (figs. 6, 7) the hemagglutination titres of both the strains tested are seen to fluctuate in the same way as in the UP-series with 5 days' incubation presented above. It is again observed that the titres of the Habel strain decreased more rapidly, in both UP-series as early as in the third passage. The titres of the Enders strain dropped below 0.3 log in both UP-series in the fourth passage. In another UP-series of Enders strain using 3 days' incubation time (fig. 17, pages 56—57) the hemagglutinin titres dropped in the fifth undiluted passage. Fluids from those passages followed by a passage with negative hemagglutination were reinoculated, each fluid into 6 embryos, and incubated now for 5 days. In none of the cases did detectable amounts of hemagglutinins appear in the pooled allantoic fluids even during this longer incubation.

Inhibitors of Hemagglutination in Allantoic Fluids from UP-series

The following experiments were made with the purpose of clarifying whether the fall in hemagglutination titres during undiluted passages was only apparent, *i.e.* only a »masking phenomenon» caused by inhibitors.

Allantoic fluids of passages giving negative hemagglutination were chosen from UP-series in which the incubation time had been 5 days. These are termed in this experiment UP-allantoic fluids.

The control allantoic fluid was prepared as follows: 0.2 ml of normal allantoic fluid from 13-day-old embryos containing 500 units of G-penicillin and 500 γ of dihydrostreptomycin per ml was injected into the allantoic cavity of eggs incubated for 8 days. The allantoic fluids were harvested after 5 days' incubation at +35°C. These are designated NN-allantoic fluids.

Two-fold dilution series of a standard virus preparation were made in UP-allantoic fluids and in NN-allantoic fluids. The dilutions were kept for one hour at room temperature, and the hemagglutination titre of each dilution was then determined.

One such experiment made with the Enders strain is presented in figure 8. It can be seen that the UP-allantoic fluid used is not a more potent inhibitor of hemagglutination by standard viruses than is the corresponding normal allantoic fluid. Similar experiments

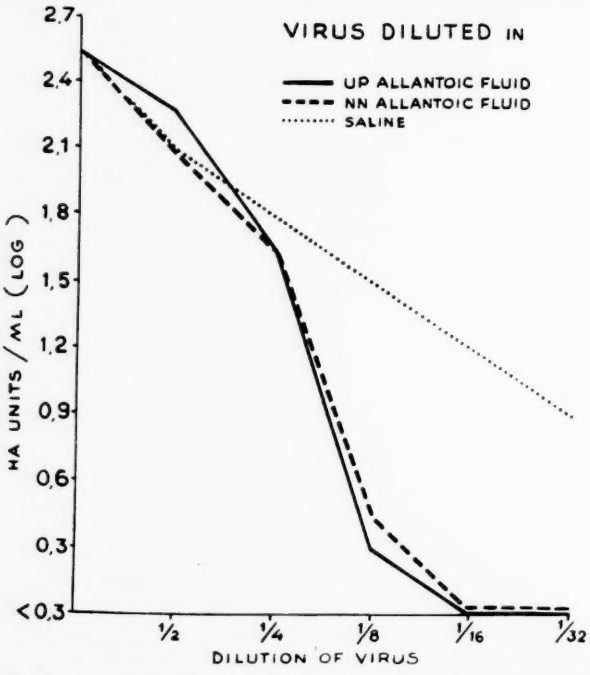

Fig. 8. — The hemagglutination inhibiting capacities of UP-allantoic fluid and normal allantoic fluid.

A virus preparation (Enders strain) was diluted in UP-allantoic fluid *), in NN allantoic fluid **), and in phosphate buffered saline in two-fold steps. The hemagglutinin titre of each dilution was determined after keeping the dilutions for one hour at room temperature.

*) UP-allantoic fluid = A nonhemagglutinating allantoic fluid preparation from UP-series of Enders strain.

**) NN allantoic fluid = Normal allantoic fluid from 13-day eggs inoculated allantoically 5 days previously with normal allantoic fluid containing antibiotics.

were also made with two other nonhemagglutinating allantoic fluids from UP-series of Habel strain. The results were similar as in the experiments reported. If the hemagglutination inhibiting capacity of these UP-allantoic fluids differed at all from the corresponding capacity of normal allantoic fluids, it was smaller rather than greater.

Several nonhemagglutinating UP-allantoic fluids were kept in a water-bath at +37°C and tested as to their hemagglutinating activity at intervals of a few hours, 1 day, and 4 days. It remained negative in all experiments.

DISCUSSION

The experimental conditions in the tests presented in figures 1 and 2, correspond to the conditions under which mumps virus is usually cultivated in laboratories. The virus fluids are stored for periods of varying length between the passages; a new passage is performed for instance when more antigen is required. The figures indicate clearly how inconstant and uncertain the yields of hemagglutinins may be if undiluted inocula are used. When using 10^{-4} diluted inocula, however, hemagglutinins were obtained very regularly.

The observation of Fazekas de St. Groth *et al.* (48) that a considerable proportion of the embryos die following a large inoculum cannot be confirmed on the basis of this study. Death of embryos was not a disturbing factor during the UP-series. It is impossible to conclude whether the different experiences are due to differences in the virus lines or eggs used or possibly to other factors.

In the complement fixation titrations the role of the V and S antigens in the titres was not analysed. It seems probable, however, that the antigen measured in the titrations consisted mainly of V antigen, *i.e.* of virus particles, because 1) all virus preparations studied were infected allantoic *fluids*, and mumps allantoic fluids have been found to contain chiefly V antigen (70, 41, 103); 2) the immune serum used had been taken three weeks after the onset of illness, when V antibodies had already developed (71, 113).

Studying the hemagglutination titres of several successive undiluted passages, a distinct regularity is revealed in the inconsistent yield of hemagglutinins. In the UP-series of Enders strain the hemagglutination titres most commonly dropped in the sixth passage if the incubation time in the individual passages was 5 days, but they already dropped in the fourth to fifth passage when incubation time was 3 days, and in the fourth passage when incubation lasted 2 days.

In the UP-series of Habel strain the hemagglutination titres decreased in the third to fourth passage if the incubation time was 5 days, but in the third passage if incubation lasted 3 or 2 days. Thus, the number of passages preceding the drop in titres tends to decrease as the incubation time in the individual passages becomes shorter.

It is clear that the drop in titres in the UP-series with 2 and 3 days' incubation cannot be due solely to the incubation time having been so short as to allow no time for measurable amounts of hemagglutinins to develop, since 1) it was also impossible to produce hemagglutinins in the negative passages of these series when extending the incubation time to 5 days; and 2) the titres invariably increased again in the later passages of the UP-series.

A study of the hemagglutinin or CF titres of the passages preceding the fall of titres in the UP-series shows that it is not possible to predict from them the size of the yield. If the inoculum is large, the yield in the UP-series must thus also be affected by factors other than the number of HA units or the amount of CF antigen in the inoculum.

The drop of titres in the UP-series can scarcely be accounted for only by a »masking phenomenon» caused by inhibitors: 1) Compared with the normal allantoic fluids, the nonhemagglutinating allantoic fluids from the UP-series contained rather less than more free inhibitors of hemagglutination; and 2) Incubation at +37°C never rendered such nonhemagglutinating allantoic fluids capable of agglutinating. If these allantoic fluids had contained large amounts of hemagglutinating viruses bound to inhibitors, one might expect the viruses to be capable of liberating themselves at +37°C.

HEMAGGLUTININ TITRES IN ALLANTOIC FLUIDS OF INDIVIDUAL EGGS

Egg variation during UP- and ST-series

In the above sections, the hemagglutinin and CF titres always represent pooled allantoic fluids from at least 6 eggs. However, in view of the fact that differences have been observed in mumps virus multiplication in individual eggs (172, 110, 61, 44, 176, 174), it seemed desirable to study the hemagglutinin titres of individual eggs during UP- and ST-series.

The results of studies of this kind on UP-series of Enders and Habel strains are given in figures 9 and 10.

The number of eggs inoculated in each passage was on an average ten. After 5 days' incubation the eggs were chilled over night. The allantoic fluid was collected from each egg by sterile technique, and its hemagglutinin titre was determined immediately. The allantoic fluids of all eggs inoculated with the same virus strain were then pooled, and the hemagglutinin titre of the pool was determined. The undiluted pool always served as the seed virus for the next passage. Storing of pools between some passages for a few days to 2 weeks at +4°C before transfer could not be avoided.

The figures show that egg variation was very slight in some passages, whereas in several passages the titres of the individual eggs differed greatly from one another.

To compare the egg variation in UP- and ST-series, 10 successive undiluted passages and 10 successive standard passages were performed in parallel, using the Enders strain.

In each passage 10 eggs on an average were inoculated with undiluted seed and an equal number of eggs with 10^{-5} diluted seed. The eggs were incubated for 5 days, after which the allantoic fluid of each egg was collected by sterile technique without previous chilling of the eggs. The hemagglutinin titre of each allantoic fluid was determined. The allantoic fluids of all eggs in the UP-series were pooled, and similarly all fluids in the ST-series. The hemagglutinin titres of the pools were determined and transfers were always made immediately.

Figure 11 illustrates the results. In the standard passages (fig. 11 A) the titres in the majority of eggs differed only less than 1 log, *i.e.* less than 10-fold. In a few passages, however, there was one egg in which the allantoic fluid did not show measurable levels of hemagglutinins. The situation in the first few undiluted

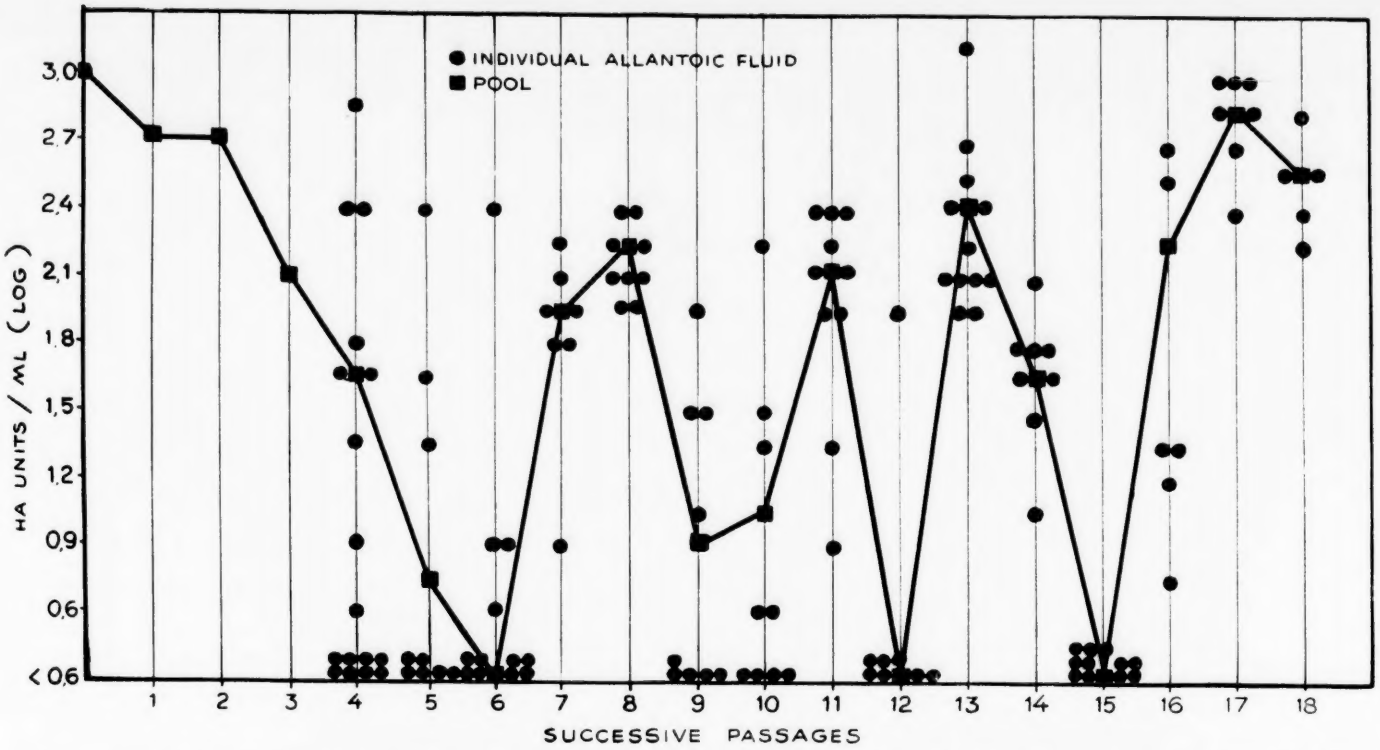

Fig. 9. — *Enders strain.* Hemagglutinin titres in allantoic fluids of individual eggs during UP-series with incubation time of 5 days in each passage. Inoculum = pooled allantoic fluid.

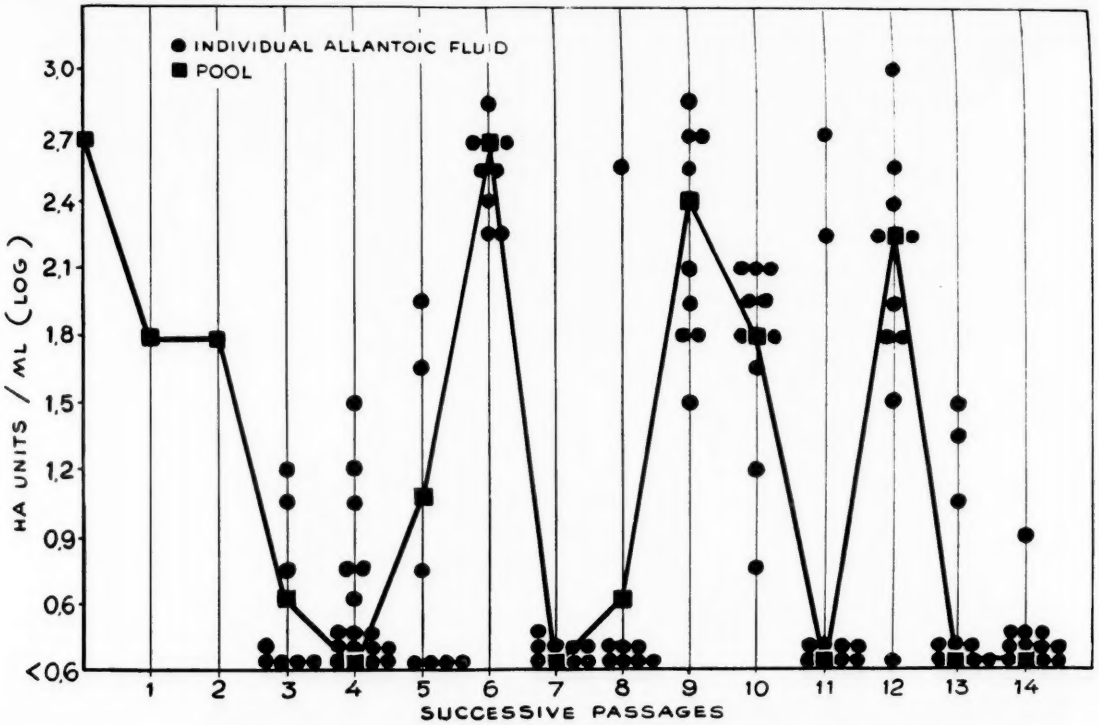

Fig. 10. — *Habel strain.* Hemagglutinin titres in allantoic fluids of individual eggs during UP-series with incubation time of 5 days in each passage. Inoculum = pooled allantoic fluid.

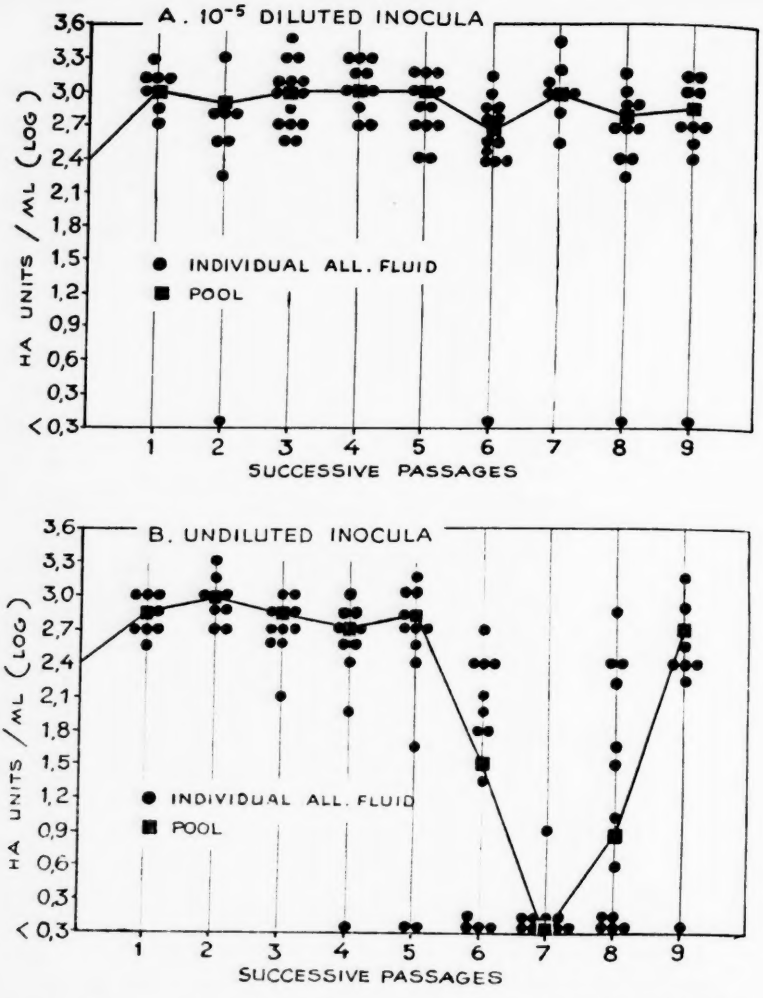

Fig. 11. — *Enders strain.* Hemagglutinin titres in allantoic fluids of individual eggs during ST-series (A.) and UP-series (B.) performed parallelly.

passages, in which the titres were still high, was similar to the standard passages (fig. 11 B). When the titres began to decrease, in the sixth undiluted passage, the titres of the individual eggs differed more than they did in the parallel standard passage. Naturally, nothing can be concluded from egg variation in the seventh passage since the titres of all eggs were less than 0.3 log, *i.e.* not measurable. When the titres again began to rise, in the eighth passage, the situation resembled the one in the sixth passage.

In view of the fact that the hemagglutinin titres of individual eggs seemed to differ greatly in the very phase when titres decreased during the UP-series, an attempt was made to study the effect of egg variation on the decrease of titres in the UP-series, as follows:

An undiluted ST-virus preparation of Habel strain was inoculated allantoically into each of 15 eggs. Following 5 days of incubation, allantoic fluids from the individual embryos were collected by sterile technique after overnight chilling and were tested individually for the presence of hemagglutinins. Three UP-series (A, B, C) were then transferred concurrently, as follows: A) Transfer was always made with the allantoic fluid having the highest titre; B) Transfer was always made with the allantoic fluid having the lowest titre; C) Transfer was always made with the pool of all the allantoic fluids. — In all these three UP-series 6 to 10 eggs were inoculated in each passage.

Concurrently with these UP-series, an ST-series was transferred using 10^{-6} diluted inocula. Six embryos were inoculated in each passage, and pooled allantoic fluid served as the inoculum.

Figure 12 shows that, in all three UP-series, the decrease in titres occurred in much the same way. Thus, egg variation did not

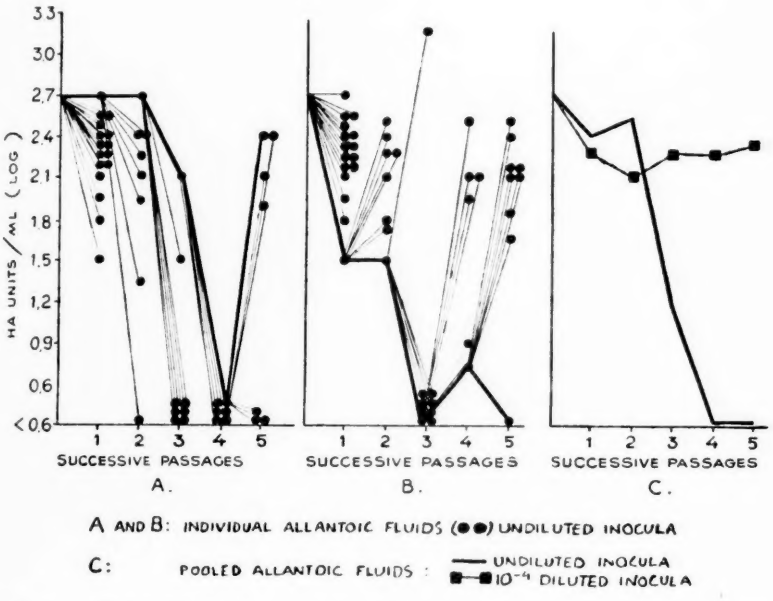

Fig. 12. — *Habel strain.* Hemagglutinin titres of individual and pooled allantoic fluids during UP- and ST-series performed parallelly.

seem to affect the drop in titres to a major extent in these experiments with the Habel strain.

In UP-series in which the hemagglutinin titres of individual eggs are given (*e.g.* figs. 9, 10, 11 B, 12 B), it was frequently observed that, while the yields in the majority of eggs were very low (less than 0.6 log), high hemagglutinin titres could be developed in one or a few individual eggs of the same passage.

Seasonal Variation in Yield of Hemagglutinins

During the progress of the studies here reported, in January and February 1958, the behaviour of the mumps virus *in ovo* was found to differ markedly from its usual behaviour. In the period stated, eggs from the same poultry farm were used as earlier, but the number of negative eggs in the standard passages was definitely greater than usual. The abnormal behaviour of the eggs was most clearly apparent when attempts were made to perform infectivity titrations. This was practically impossible during the period mentioned, because negative eggs, *i.e.* eggs in which measurable levels of hemagglutinins did not develop, were so numerous that a clearcut end-point could not be determined. Surprisingly enough, a sharp end-point was obtained a few times in infectivity titrations during this period too: the batch of eggs then used did not, in fact, contain more than the usual number of negative eggs. In March 1958 the eggs, still obtained from the same poultry farm, did again begin to behave »normally».

DISCUSSION

In some standard passages a small percentage of the allantoic fluids failed to show measurable levels of hemagglutinins (fig. 11 B). This can scarcely be explained by error in inoculation, since the same phenomenon appeared also in Lundbäck's experiments (111), in which allantoic inoculation took place under direct visual control. Lundbäck moreover noted that the relative number of such eggs decreased with an increase in the size of inoculum, in other words hemagglutinins developed most regularly in the allantoic fluids of

individual eggs when the inocula were large. The results of the present study indicate, however, that when a large inoculum was used for several successive passages, the egg variation distinctly increased in some passages (figs. 9, 10, 11 B). This cannot be due to »bad» batches of eggs alone, because concurrent standard passages in eggs of the same batch showed definitely less egg variation.

COMPARISON BETWEEN INFECTIVITY AND HEMAGGLUTININ TITRES DURING UP- AND ST-SERIES

In all the above experiments the hemagglutination or complement fixation titres served as measures of virus material. In this section, the infectivity titre — the most sensitive criterion of living viruses — will be used during undiluted and standard passages.

Growth Curve after Inoculation of Undiluted ST-seed

Each of 50 chick embryos incubated for 8 days were inoculated allantoically with 0.5 ml of undiluted standard virus allantoic fluid (Enders strain), containing $10^{8.7}$ EID_{50} and $10^{2.4}$ HA units per inoculum. The eggs were incubated at +35°C. After 3 hours, 1 day, 2, 3, 4, 5, 6 and 7 days the eggs were sacrificed in batches of 6, and the allantoic fluids harvested after chilling the eggs for a few hours at +4°C. The allantoic fluids of all 6 eggs were pooled and the hemagglutinin and infectivity titres of the pool were determined. All hemagglutination titrations were performed in duplicate. In the case of some pools, several infectivity titrations were performed, the fluids having been stored at +4°C for at most about a week between titrations.

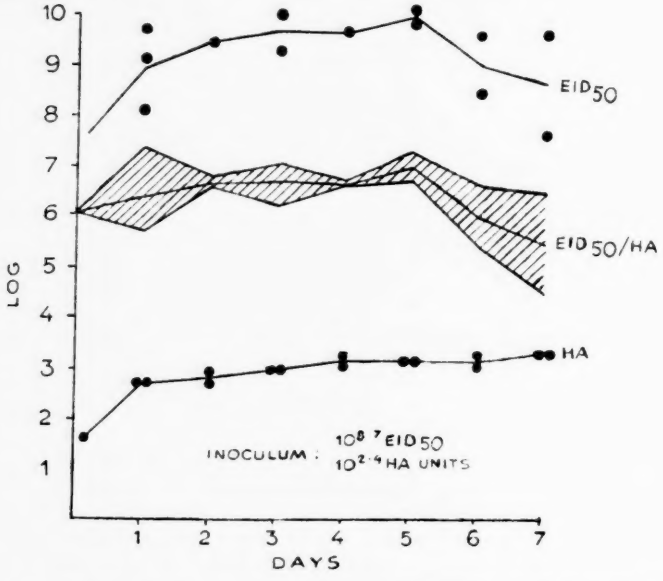

Fig. 13. — *Enders strain.* Development of infectivity and hemagglutinins in allantoic fluids after inoculation of undiluted ST-seed.

The results are given in figure 13. This experiment was the only one that was made in part during the »bad season» described above (*cp.* page 50). This probably accounts for the fact that the results of different infectivity titrations of the same virus preparation differed more than usual (*cp.* page 32). In spite of the infectivity titres being thus more inaccurate than usually, the results seem to allow the following statement:

Both the hemagglutinin and the infectivity titre increase in one day almost to the maximum. Up to the fifth day the infectivity titres increase rather than decrease. Thereafter, the infectivity titres show a decreasing tendency on the sixth and seventh days, the hemagglutination titres however are maintained at a maximum. The EID_{50}/HA ratio (the ratio between the infective and hemagglutinin titres of a virus preparation) varies from 6.3 to 7.0 (log) between the first and the fifth day. On the sixth and seventh days the EID_{50}/HA shows a decreasing tendency.

Infective and Hemagglutinin Titres in UP- and ST-series with Incubation Time of Five Days in Each Passage

In each undiluted and standard passage the average number of eggs inoculated was 10. After incubation for 5 days, the allantoic fluids were collected without previous chilling of the eggs, and the fluids of all eggs were pooled. Immediately upon this, the hemagglutinin and infective titres of the pooled fluids were determined. Transfers were carried out without storing the fluids between passages. The volume of the inoculum was 1.0 ml in both the UP- and the ST-series. In the standard passages the inoculum was diluted 10^{-6}.

Some nonhemagglutinating allantoic fluids of the UP-series were concentrated as follows: 20 ml of these UP-allantoic fluids and an ST-virus preparation were ultracentrifuged (Spinco model L) for 45 minutes at 25,000 r.p.m. The supernatants were removed with utmost care using a Pasteur pipette. The fluids were made up to 1.0 ml with phosphate buffered saline (pH 7.2). The hemagglutinin titre of the standard virus preparation had increased about 1 log, and concentration was thus considered about 10-fold.

Figure 14 is a presentation of 12 successive undiluted and standard passages. It can be seen that in the standard passages the infectivity titres were invariably very high. The lowest titre

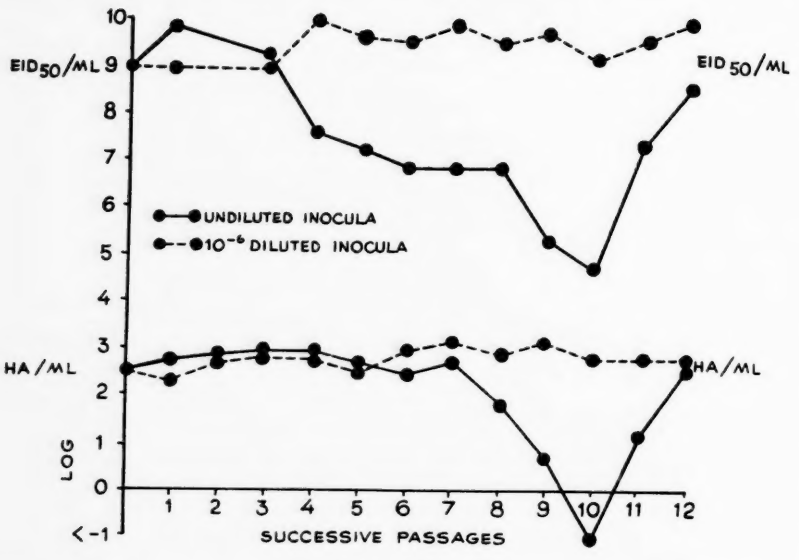

Fig. 14. — *Enders strain.* Infectivity (EID_{50}) and hemagglutinin (HA) titres in allantoic fluids during UP-series and ST-series performed parallelly. Incubation time in each passage *5 days.*

was 9.0 and the highest 10.0 (The UP-series was atypical in that the hemagglutinin titre did not decrease until the ninth to tenth passage, whereas it usually decreased with the Enders strain in the sixth passage when the incubation time was 5 days in each passage.) The infectivity titres were high in the first three undiluted passages, of the same order as in the standard passages. However, in the undiluted passages following next, the infectivity titres began to decrease, reaching a minimum in the tenth passage, as did the hemagglutinin titres. The highest infectivity titre in the UP-series was 9.7 and the lowest 4.7. Thus the difference between individual infectivity titres was maximally 5 log during the UP-series.

Closer study of the EID_{50}/HA ratio during the ST- and UP-series of the experiment presented in figure 14 showed the following findings: In the ST-series the EID_{50}/HA ratio was fairly constant in all passages (fig. 15): the highest value was 7.2 and the lowest 6.15; the mean was 6.66 and the standard deviation 0.3. In the first few passages of the UP-series the EID_{50}/HA ratio decreased about 2 log, remaining between 4 and 5 up to the ninth passage. In the tenth passage no precise figure for the EID_{50}/HA ratio

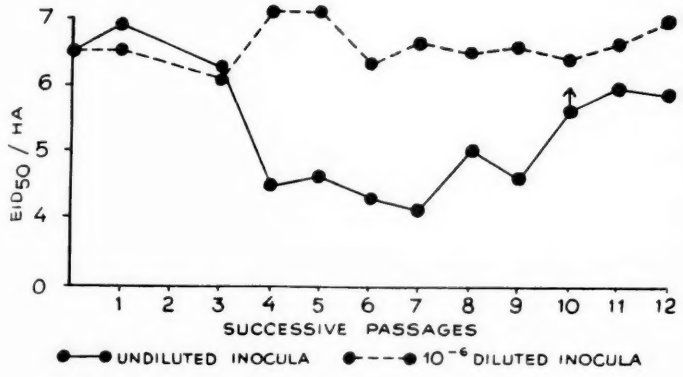

Fig. 15. — EID_{50}/HA (infectivity-hemagglutinin) ratio during UP- and ST-series presented in Fig. 14.

could be determined because hemagglutination was negative. Considering that the allantoic fluid of the tenth passage did not show agglutinating activity even by 10-fold concentration by centrifugation, the EID_{50}/HA value in this passage must be at least about 1 log higher than the corresponding infectivity titre,

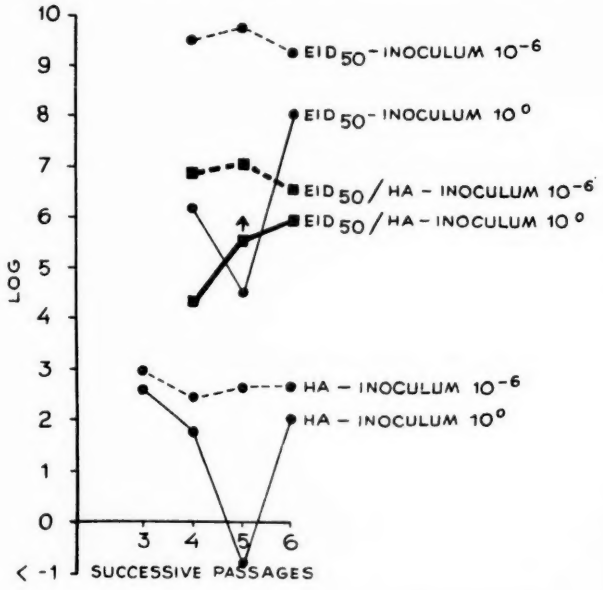

Fig. 16. — *KS strain.* Infectivity (EID_{50}) and hemagglutinin (HA) titres and infectivity-hemagglutinin ratios (EID_{50}/HA) in allantoic fluids during UP- and ST-series performed parallelly. Incubation time in each passage *5 days.*

i.e. it must at least exceed 5.7. With an increase in infectivity and hemagglutination titres in the eleventh and twelfth passages, the EID_{50}/HA value also rises, approaching the corresponding values in the ST-series. Assuming that the dispersion of an individual EID_{50}/HA value in the UP-series is the same as in the ST-series, *viz.* 0.3, the EID_{50}/HA values of the fourth to ninth undiluted passages, calculated on the basis of Student's t-test (31), are highly significantly lower than the mean of EID_{50}/HA values in the ST-series.

The EID_{50}/HA value was studied also in UP- and ST-series of KS strain, the method being exactly the same as in the experiment described above. The results (fig. 16) were in keeping with those obtained with the Enders strain. The infectivity titres and the EID_{50}/HA ratio in the standard passages were very similar to those of the Enders strain. In the undiluted passage preceding the decrease in titres, the EID_{50}/HA ratio also in this experiment was well over 2 log lower than in the standard passages. The EID_{50}/HA ratio increased clearly with a decrease in titres, although the ratio could not be precisely defined as the allantoic fluid from the fifth passage did not show hemagglutinating activity even by 10-fold concentration.

Infective and Hemagglutinin Titres in UP-series with Incubation Time of Three Days in Each Passage

In the following experiment regarding the variation of the EID_{50}/HA ratio during undiluted passages of the Enders strain, the method differed from the one described in the preceding section, as follows: 1) At least 20 nine-day-old embryos were inoculated in each passage with 0.5 ml of seed virus; 2) In each passage the incubation time was 3 days; 3) The allantoic fluid of each egg was assayed individually, undiluted, for the presence of hemagglutinins. Only positive allantoic fluids were pooled. The hemagglutination and infectivity titres of the pool were determined and the transfer was carried out with the pool.

The results are given in figure 17. In the first four passages, hemagglutinins exceeded the level of detectability in practically

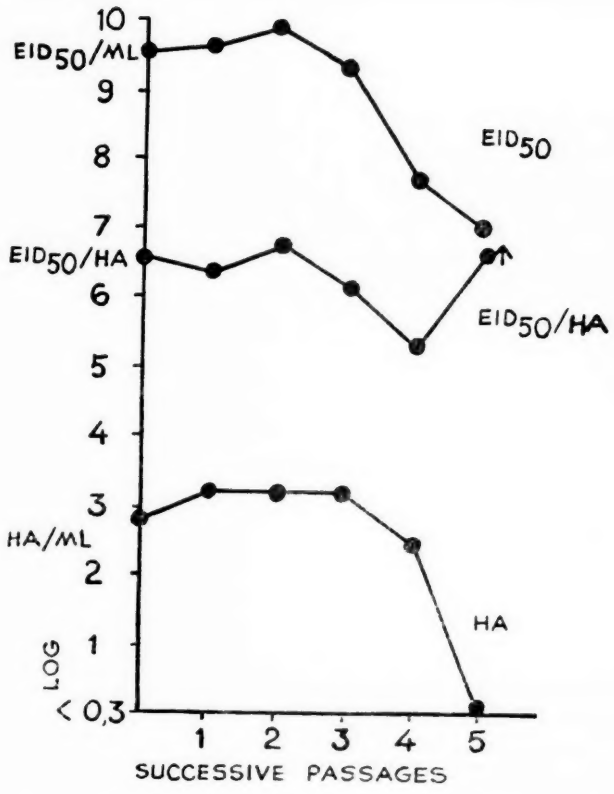

Fig. 17. — *Enders strain.* Infectivity (EID_{50}) and hemagglutinin (HA) titres and infectivity-hemagglutinin ratios (EID_{50}/HA) in allantoic fluids during UP-series with incubation time of *3 days* in each passage.

speaking all the eggs, but in the fifth passage the reaction was negative in all the eggs. The experiment was continued by inoculating allantoic fluid of the fourth passage into a further group of 15 eggs, incubation now lasting 6 days. (Before inoculation, the fluid had been kept for 3 days at +4°C.) Even this extended incubation failed to produce detectable amounts of hemagglutinins in any of the eggs. During this UP-series the infectivity titres decreased slightly less than 3 log. The EID_{50}/HA value in the first two undiluted passages was of exactly the same order as earlier noted in standard passages of the Enders strain. In the third passage the EID_{50}/HA value was already at the lower limit of the range of fluctuation of this value in the standard passages. In the

fourth passage this ratio fell 1.4 log below the mean for the ST-series given above. Statistically this difference is highly significant, assuming that the dispersion of an individual titre was 0.3 in the UP-series as it was both in the ST-series presented above (fig. 14) and in the repeated titrations of the one ST-virus preparation (page 32).

DISCUSSION

The infectivity titres of the ST-virus preparations were clearly higher than those obtained by most other investigators with mumps virus preparations (106, 55, 66, 61, 111, 92, 88, 151, 14). Only Weil *et al.* (172) reported nearly as high values. In Finland, however, infectivity titres about equally high as in the present work have been obtained previously with mumps virus allantoic fluids (136). The infectivity titres of standard virus allantoic fluids were invariably from 10^9 to 10^{10} during the present investigation. The titre 10^{10} was obtained several times in allantoic fluids infected with Enders strain.

The method of assaying infectivity titres used in the present study may have been more sensitive than those generally employed. For example the following factors may have been of importance: 1) The inoculation was always made as soon as possible after preparation of the dilution series. Before that the inocula were always kept in dark. Skinner & Bradish (150) found that highly significant reductions in the infectivity titres may be obtained with several viruses if the inocula are kept for one hour or even less in normal laboratory daylight or artificial light after preparing the dilution series. 2) The incubation time was 6 days. It has been observed that, with incubation for 6 days, about 0.5 log higher mumps virus infectivity titres are obtained than with 5 days' incubation (61). 3) Selected, »sensitive» fowl cells (*cp.* page 29) were always used in testing for the presence of hemagglutinins. 4) The allantoic fluids were not diluted in these assays for hemagglutinins, because it was found that the hemagglutinin levels in the allantoic fluids of some eggs could be rather low (*cp.* figs. 9, 10, 11, 12, 18, 19, and 20).

On the other hand, it appears possible that the high infectivity titres were due at least in part to the fact that the virus had become

very well adapted to the allantoic cavity of the chick embryo in the course of several dozens of consecutive standard passages: it was noted that the hemagglutinin titres showed a rising tendency during the first score or so of standard passages performed in the work.

In the studies of Isaacs & Donald (92) the EID_{50}/HA ratios of the mumps virus preparations varied considerably. In the present work, however, the EID_{50}/HA values of the individual ST-virus preparations were fairly constant: the variations were not greater than might be expected on the basis of the »standard deviation» of the infectivity titres (page 32). The EID_{50}/HA values of the ST-viruses in the present study were clearly higher than those generally reported for mumps virus preparations (55, 168, 92, 111), but fairly similar to the corresponding ratios obtained with influenza A, B and D virus and Newcastle disease virus (44 a, 93).

According to Isaacs & Donald (92), one agglutinating unit as determined by the pattern method corresponds to about $10^{7.5}$ mumps virus particles counted by electron microscopic means. In the work here reported, the hemagglutinin titres were determined with a method highly similar to that used by Isaacs & Donald. Supposing that, in the present work too, one agglutinating unit corresponds to about $10^{7.5}$ virus particles, the number of virus particles per EID_{50} can be calculated. In the standard passages of Enders strain the EID_{50}/HA ratio was on an average 6.66. The ratio of virus particles to EID_{50} would thus average 0.85 log, or in other words one infective dose would correspond on the average to about 7 virus particles of Enders strain. Isaacs & Donald, however, in their studies calculated that the number of mumps virus particles per EID_{50} was several hundreds (92).

During the first few passages of the UP-series the EID_{50}/HA value was of the same order as it was with standard viruses (figs. 13, 15, 17). In the UP-series of Enders strain in which the incubation time in the passages was 5 days, the EID_{50}/HA ratio decreased in the fourth passage; in the third passage it was still as high as the average ratio of ST-viruses (fig. 15). In the UP-series in which the incubation time in the passages was 3 days, the EID_{50}/HA ratio was perhaps already slightly lower in the third passage, and in the fourth passage it was highly significantly lower than in

the case of ST-viruses (fig. 17). Thus, whether the incubation time was 3 or 5 days in the individual passages, an equal number of successive undiluted passages preceded the decrease in the EID_{50}/HA value. This question will be reverted to in the General Discussion when dealing with the causes of the decrease in the EID_{50}/HA ratio in the UP-series.

INTERFERENCE PHENOMENA

»Autointerfering» Activity of UP-virus Preparations

Figure 18 illustrates a UP-series of Enders strain in which incubation time in each passage was 5 days. The hemagglutinin titres fell below 0.6 in the seventh passage. Inoculation of undiluted allantoic fluid from the sixth passage did not cause detectable levels of hemagglutinins to form in any of the 10 eggs inoculated.

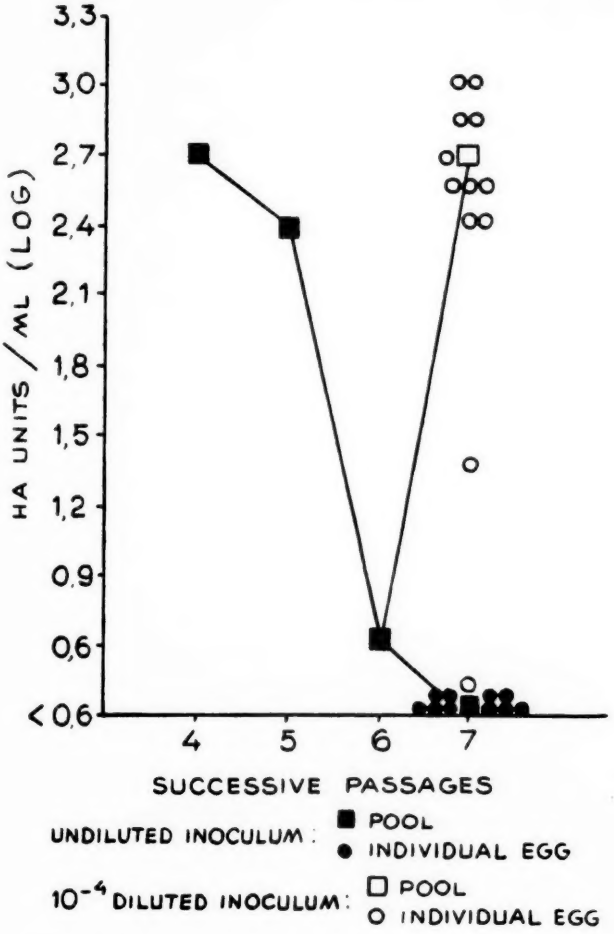

Fig. 18. — »Autointerference». Increase in hemagglutinin yield with dilution of inoculum derived from UP-series (Enders strain).

But when the same allantoic fluid from the sixth passage was inoculated diluted 10^{-4}, high hemagglutinin titres developed within 5 days of incubation in the great majority of the eggs. The case was thus one of »autointerference». In many different UP-series the observation was invariably the same, *i.e.* the decrease in hemagglutinin titres could be avoided by diluting the inoculum.

The following method was used in an endeavour to study this »autointerference» more closely:

Allantoic fluids derived from the passage preceding the fall in titres were selected from the different UP-series. Prior to pooling, the allantoic fluids of individual eggs were tested for the presence of hemagglutinins, and only the agglutinating allantoic fluids were pooled. Ten-fold dilution series were prepared from these pooled allantoic fluids, and each dilution was inoculated into 6 to 10 eggs. After incubation for 5 days the allantoic

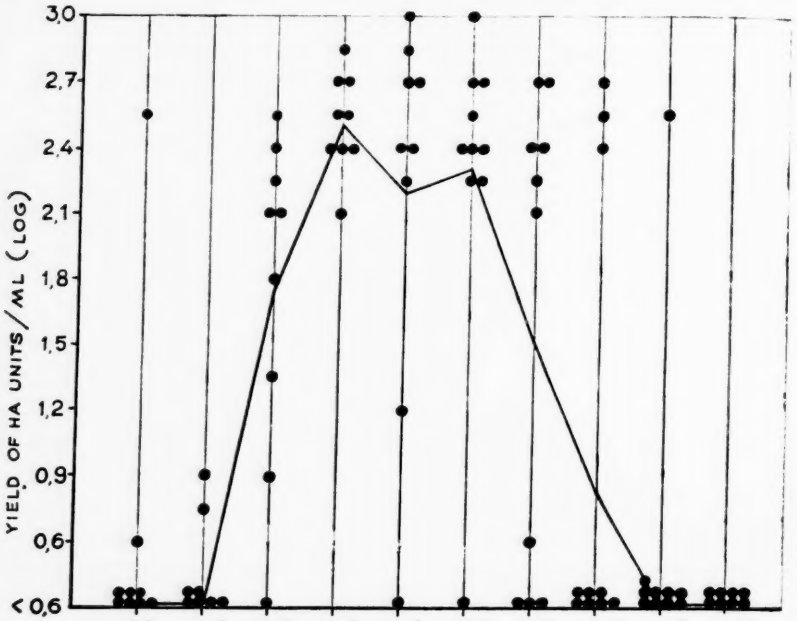

Fig. 19. — »Autointerfering» property of a UP-allantoic fluid (Enders strain). Undiluted inoculum = $10^{0.8}$ HA units and $10^{5.7}$ EID_{50}.

A ten-fold dilution series of the UP-allantoic fluid was prepared. 0.2 ml of each dilution was inoculated allantoically into 6 to 10 eggs. After incubation for 5 days the hemagglutinin titres of individual allantoic fluids were determined.

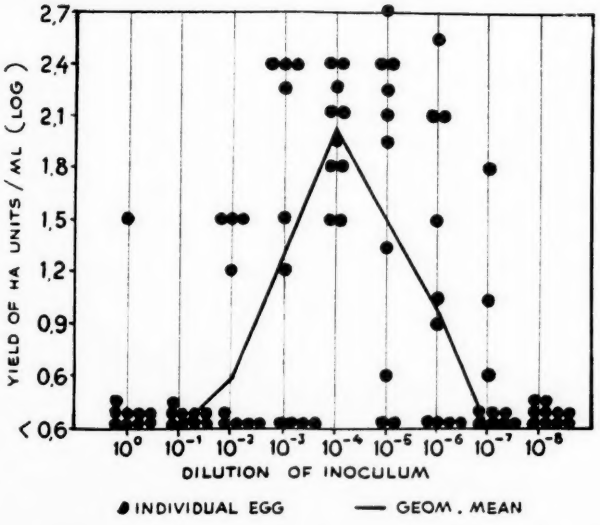

Fig. 20. — »Autointerfering» property of a UP-allantoic fluid (Habel strain). Undiluted inoculum = $10^{1.4}$ HA units and $10^{5.6}$ $\overline{EID}_{50}$.

A ten-fold dilution series of the UP-allantoic fluid was prepared. 0.2 ml of each dilution was inoculated allantoically into 10 eggs. After incubation for 5 days the hemagglutinin titres of individual allantoic fluids were determined.

fluids were harvested individually; their hemagglutinin titres were determined or, in some experiments, only undiluted allantoic fluids were tested for the presence of hemagglutinins.

One such experiment is illustrated in figure 19. The allantoic fluid inoculated was derived from a UP-series of Enders strain in which the incubation time was 5 days in each passage. The hemagglutinin titre of this allantoic fluid preparation was $10^{1.5}$ and the infectivity titre $10^{6.4}$. Thus, when the volume of the inoculum was 0.2 ml, the undiluted inoculum contained $10^{0.8}$ HA units and $10^{5.7}$ EID_{50}. The figure shows that in the great majority of eggs no measurable hemagglutinin levels developed as a result of the undiluted inoculum. A phenomenon already referred to (page 50) was again observed, *i.e.* that high hemagglutinin titres developed in one egg as contrasted with the others. With an inoculum diluted 10^{-1} (= $10^{-0.2}$ HA units), the yield of hemagglutinins was also poor. An inoculum diluted 10^{-2} (= $10^{-1.2}$ HA units) resulted in highly varying hemagglutinin levels in individual eggs. Not until 10^{-3} diluted inocula were used did high titres develop in all eggs. With further diminution of the inoculum,

when approaching the infectivity end-point, the number of negative eggs of course increased again.

Figure 20 shows a similar experiment with the Habel strain. The UP-virus allantoic fluid was derived from a UP-series in which the incubation time was 5 days in each passage. It contained $10^{1.4}$ HA units and $10^{5.6}$ EID_{50} per undiluted inoculum. Inocula diluted 10^{0}—10^{-1} (= $10^{1.4}$—$10^{0.4}$ HA units) resulted in very poor yields of hemagglutinins. 10^{-2}—10^{-3} diluted inocula (= $10^{-0.6}$—$10^{-1.6}$ HA units) produced detectable amounts of hemagglutinins in as little as about 50 per cent of the eggs. Only a 10^{-4} diluted inoculum produced measurable hemagglutinin levels in all the eggs.

The »autointerfering capacity of another UP-virus allantoic fluid of Habel strain is presented in table 3. From an inoculum

TABLE 3

»AUTOINTERFERING« PROPERTY OF A UP-ALLANTOIC FLUID (HABEL STRAIN)

Inoculum		Yield
Dilution	HA Units	
10^{0}	$10^{1.1}$	0/10 [1]
10^{-1}	$10^{0.1}$	0/6
10^{-2}	$10^{-0.9}$	3/6
10^{-3}	$10^{-1.9}$	3/6
10^{-4}	$10^{-2.9}$	5/6

[1] Numerator = number of eggs containing detectable levels of hemagglutinins after 5 days' incubation.
Denominator = total number of eggs inoculated.

TABLE 4

«AUTOINTERFERING» PROPERTY OF A UP-ALLANTOIC FLUID (ENDERS STRAIN)

Inoculum		Yield
Dilution	HA Units	
10^{0}	$10^{2.1}$	0/6 [1]
10^{-1}	$10^{1.1}$	0/6
10^{-2}	$10^{0.1}$	3/7
10^{-3}	$10^{-0.9}$	6/7
10^{-4}	$10^{-1.9}$	6/6

[1] Numerator = number of eggs containing detectable levels of hemagglutinins after 5 days' incubation.
Denominator = total number of eggs inoculated.

diluted 10^{-1} (= $10^{0.1}$ HA units) detectable levels of hemagglutinins developed in none of the eggs, and also from 10^{-2} and 10^{-3} diluted inocula (= $10^{-0.9}$ and $10^{-1.9}$ HA units) only in 50 per cent of the eggs.

The virus allantoic fluids in the above experiments were all from UP-series in which the incubation time was 5 days in each passage. Table 4 is based on an »autointerference» study with UP-virus allantoic fluid of Enders strain derived from a UP-series in which the incubation time of 3 days was employed in each passage. A 10^{-1} diluted inoculum (= $10^{1.1}$ HA units) did not give rise to measurable hemagglutinin levels: a 10^{-2} diluted inoculum (= $10^{0.1}$ HA units) produced detectable hemagglutinin levels in slightly less than 50 per cent of the eggs.

Interfering Capacity of UP-virus Preparations and of Inactivated Virus

The interfering capacity of UP allantoic fluids was studied in the experiments to be reported below by inoculating the same eggs with both UP- and ST-virus allantoic fluid and by comparing the yields with controls.

UP = Allantoic fluid preparation of the passage preceding the fall in hemagglutinin titres in UP-series.

NN = Pooled allantoic fluid from 13-day eggs inoculated allantoically 5 days previously with normal allantoic fluid (13-day eggs) containing 500 units of G-penicillin and 500 γ of dihydrostreptomycin.

ST = Standard virus allantoic fluid = challenge virus.

In each experiment a number of 8-day eggs were inoculated allantoically with 0.2 ml of UP or NN. A group of the UP-eggs received no other inoculation, but served as UP-controls. All other eggs, both the UP- and the NN-eggs, were inoculated allantoically immediately after this or after a few hours with 0.2 ml of suitably diluted ST containing 2, 4, 6 or 8 log EID_{50}. The eggs were incubated for 5 days at +35°C. After this the allantoic fluids were collected individually and tested undiluted for the presence of hemagglutinins.

The results of an experiment of this kind are given in table 5 (A, B and C). It will be seen (A) that $10^{1.7}$ HA units of UP-virus allantoic fluid of Habel strain inhibited a challenge dose of 10^6 EID_{50}, inoculated simultaneously, from producing measurable amounts of

TABLE 5 (A. B. C.)

INTERFERING CAPACITY OF SOME UP-ALLANTOIC FLUIDS

	INOCULUM	YIELD
	UP Control	1/6[1]
A. *Habel strain*	NN + ST (10^8 EID_{50})	4/4
	NN + ST (10^6 EID_{50})	5/5
	NN + ST (10^4 EID_{50})	6/6
UP = $10^{1.7}$ HA units per inoculum	NN + ST (10^2 EID_{50})	6/6
Challenge dose (ST) was inoculated	UP + ST (10^8 EID_{50})	4/4
simultaneously with NN and with UP	UP + ST (10^6 EID_{50})	1/5
	UP + ST (10^4 EID_{50})	1/6
	UP + ST (10^2 EID_{50})	0/6
	UP Control	0/10
	NN + ST (10^7 EID_{50})	6/8
	NN + ST (10^5 EID_{50})	8/9
B. *Habel strain*	NN + ST (10^3 EID_{50})	5/7
UP = $10^{0.5}$ HA unit per inoculum	NN + ST (10^1 EID_{50})	5/8
Challenge dose (ST) was inoculated	UP + ST (10^7 EID_{50})	9/10
16 hours after NN and UP	UP + ST (10^5 EID_{50})	2/12
	UP + ST (10^3 EID_{50})	0/9
	UP + ST (10^1 EID_{50})	0/9
	UP Control	1/11
	NN + ST (10^8 EID_{50})	9/9
	NN + ST (10^6 EID_{50})	9/9
C. *Enders strain*	NN + ST (10^4 EID_{50})	10/10
UP = $10^{1.1}$ HA units per inoculum	NN + ST (10^2 EID_{50})	8/9
Challenge dose (ST) was inoculated	UP + ST (10^8 EID_{50})	0/10
14 hours after NN and UP	UP + ST (10^6 EID_{50})	0/10
	UP + ST (10^4 EID_{50})	1/9
	UP + ST (10^2 EID_{50})	0/9

[1] Numerator = number of eggs containing detectable levels of hemagglutinins after 5 days' incubation.
Denominator = total number of eggs inoculated.

NN = Pooled allantoic fluid from 13-day eggs inoculated allantoically 5 days previously with normal allantoic fluid containing antibiotics.
UP = Allantoic fluid preparation from UP-series. All the UP-preparations employed in this experiment were known not to produce detectable hemagglutinin levels when using undiluted inoculum.
ST = Standard virus preparation = challenge virus.

hemagglutinins during 5 days of incubation. However, this UP-virus allantoic fluid did not, by the method used, demonstrably inhibit a challenge dose of 10^8 EID_{50}.

$10^{0.5}$ HA units of another UP-virus allantoic fluid of Habel strain was capable of inhibiting the multiplication of a challenge dose of 10^5 EID_{50} inoculated 16 hours later, but no longer inhibited the multiplication of a challenge dose of 10^7 EID_{50} (B).

A UP-virus allantoic fluid of Enders strain seemed to have an even stronger interfering capacity (C). $10^{1.1}$ HA units of this UP-virus preparation clearly still interfered with a challenge dose of 10^8 EID_{50} inoculated 14 hours later.

The role of heat-inactivated viruses must be taken into account in dealing with the nature of the phenomena appearing during UP-series. For this reason the interfering capacity of *in vitro* at +35°C inactivated ST-viruses (=Δ ST-viruses) was studied.

Pooled allantoic fluid derived from a standard passage of Enders strain (EID_{50}/ml $10^{9.0}$ and HA/ml $10^{2.4}$) was placed immediately after harvesting at +35°C in a rubber stoppered flask. On the basis of previous experiments (26) it was known that the infectivity of such a virus preparation under these conditions disappears in about 7 to 10 days.

(The half-life of the infectivity of mumps virus (Enders, Habel, KS strains) in allantoic fluid is 7 to 8 hours at +35°C *in vitro*, according to the investigations of the present author (26)).

ST-virus allantoic fluids kept *in vitro* at +35°C for 7 and 10 days were therefore used in this experiment. For the purposes of this study these are termed Δ ST (7 days) and Δ ST (10 days). The hemagglutination titre of both Δ ST (7 days) and Δ ST (10 days) was $10^{2.4}$, *i.e.* the same as in the original ST-virus allantoic fluid before the inactivation.

NN = Pooled allantoic fluid from 13-day embryos inoculated 5 days previously with normal allantoic fluid (13 day embryos) and containing antibodies as above.

ST = Standard virus allantoic fluid = challenge virus.

A number of 8-day-old embryos were inoculated allantoically with 0.2 ml of Δ ST or NN. A group of the Δ ST embryos received no other inoculation but served as Δ ST controls. After 3 hours all other embryos, both the Δ ST and the NN embryos, were inoculated allantoically with 0.2 ml of suitably diluted ST containing 3 or 6 log EID_{50}. The eggs were incubated for 5 days at +35°C. The allantoic fluids were then collected individually and tested undiluted for the presence of hemagglutinins.

TABLE 6

INTERFERING CAPACITY OF INACTIVATED MUMPS VIRUS (= Δ ST)

Δ ST = $10^{1.7}$ HA units per inoculum (0.2 ml). Challenge dose (ST) was inoculated *3 hours* after NN and UP

INOCULUM	YIELD
Δ ST (7 days) control	3/12 [1]
NN + ST (10^6 EID_{50})	4/5
NN + ST (10^3 EID_{50})	4/4
Δ ST (7 days) + ST (10^6 EID_{50})	5/8
Δ ST (7 days) + ST (10^3 EID_{50})	7/8
Δ ST (10 days) control	1/10
NN + ST (10^6 EID_{50})	6/8
NN + ST (10^3 EID_{50})	5/7
Δ ST (10 days) + ST (10^6 EID_{50}).....	6/6
Δ ST (10 days) + ST (10^3 EID_{50}).....	5/7

[1] Numerator = number of eggs containing detectable levels of hemagglutinins after 5 days' incubation.
Denominator = total number of eggs inoculated.

NN = Pooled 'allantoic' fluid from 13-day eggs inoculated allantoically 5 days previously with normal allantoic fluid containing antibiotics.

ST = Virus preparation ($10^{9.0}$ EID_{50} and $10^{2.4}$ HA/ml) inactivated for 7 days (Δ ST (7 days)) or for 10 days (Δ ST (10 days)) at + 35°C *in vitro*.

ST = Standard virus preparation = challenge virus.

The results are seen in table 6 (A and B). The Δ ST controls show that the ST-virus allantoic fluid inactivated for 7 days *in vitro* at +35°C produced measurable amounts of hemagglutinins in one fourth of the embryos, while the same fluid inactivated for 10 days *in vitro* at +35°C produced measurable amounts of hemagglutinins in one tenth of the embryos. Using $10^{1.7}$ HA units of the Δ ST-virus allantoic fluids, neither of them showed any interfering activity by the method employed in the present work.

DISCUSSION

The presence of hemagglutinins was the criterion of viruses in all interference assays. Even though hemagglutination had been negative in all eggs, the interference was certainly never complete: 1) The studied allantoic fluids derived from non-hemagglutinating passages of UP-series always contained at least 10^4 infectious doses (figs. 14, 16, 17); 2) The virus strain was never

lost in undiluted passages, the titres always rose again. For greater simplicity, however, the interference will be termed »complete» if the development of measurable levels of hemagglutinins was inhibited in all or in the great majority of eggs. The interference is termed »partial» if about 50 per cent of the eggs gave no positive hemagglutination or if the titres were clearly lower than those in control embryos.

In studying the UP-virus allantoic fluids of Habel strain the lowest numbers of hemagglutinating units capable of inducing »complete» interference were $10^{0.1}$ (table 3), $10^{0.4}$ (fig. 20) and $10^{0.7}$ (table 5 B). The corresponding number in the case of Enders strain was $10^{-0.2}$ (fig. 19). For inducing »partial» interference, only $10^{-1.6}$ (fig. 20) and $10^{-1.9}$ (table 3) HA units were required using the Habel strain, and $10^{-1.2}$ HA units using the Enders strain (fig. 19). If the dose of challenge virus increased sufficiently, no interference could any longer be demonstrated (table 5 A and B).

The interference studies with ST-viruses inactivated *in vitro* at +35°C showed (table 6 A and B) that $10^{1.7}$ HA units of Δ ST-viruses were not capable of even »partially» interfering against a challenge dose of 10^3 EID_{50}. This can hardly be accounted for by overinactivation of the Δ ST-viruses, since the Δ ST-virus allantoic fluids had not even lost all of their infectivity. Thus, Δ ST-viruses could not be shown to possess any interfering capacity with the method here used. In any case, the results seem to show that the interfering capacity of ST-virus allantoic fluids inactivated *in vitro* at +35°C is less than 1/1000th of the interfering capacity of UP-virus allantoic fluids *in relation to the hemagglutinin titres.*

Assuming that one HA unit corresponds to about $10^{7.5}$ mumps virus particles (92) and that the number of allantoic cells in an 8-day-old chick embryo is $10^{7.3}$ (164, 25), the multiplicities (= the number of virus particles in the inoculum per host cell) required for interference can be calculated. Thus calculated, not even »partial» interference is produced at the multiplicity of about 80 Δ ST-viruses. However, »complete» interference is induced at the multiplicities of 1, 2, 4 and 20 (different experiments) UP-virus particles, and »partial» interference is induced at the multiplicities of 0.02, 0.04, 0.1 and 2 (different experiments) UP-virus particles. The last figures are strikingly low. An attempt is made to account for this problem in the General Discussion.

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GENERAL DISCUSSION

1. Effect of Dilution of the Inoculum on the Yield

Study of the virus yields in the UP-series establishes beyond doubt that the use of undiluted inocula when passing mumps virus allantoically in chick embryos has adverse effects. The results here presented also show distinctly that the inferiority of the undiluted inoculum compared with 10^{-4}—10^{-6} diluted inoculum does not become apparent until both kinds of inocula are compared during several successive passages.

On the basis of the above results the unexplained poor yields frequently obtained by some investigators might readily be explained by the fact that the serial passages had been performed with undiluted inocula (9, 154).

Weil *et al.* noted (172) that the yields of hemagglutinins in the allantoic fluids were about equally good when using seeds diluted from 10^{0} to 10^{-3}. From this they concluded that the concentration of the virus in the inoculum has no apparent effect on the growth of the virus in the eggs. Nor was it ever observed in the present study that, when starting UP- and ST-series with fresh ST-allantoic fluid, the yields with undiluted inocula were poorer than with 10^{-4}—10^{-6} diluted inocula, rather the reverse. However, on the basis of the findings here obtained, the conclusion of Weil *et al.* is no longer tenable after a few undiluted passages.

The titres of Enders strain (EID_{50}, HA and CF) generally decreased in the sixth undiluted passage when the incubation time in individual passages was 5 days. This calls to mind the early work of Rocchi (141) in which lesions could never be carried beyond the sixth passage when cultivating mumps virus on the chorio-allantoic membrane. It has later been questioned whether the

lesions described by Rocchi were due to mumps virus (155). If so, it is perhaps not impossible that the disappearance of the lesions in serial passages was caused by the fact that the passages were performed with undiluted inocula.

According to the studies of Lundbäck (111), the best and most constant mumps virus yields are obtained when using 10^{-2}—10^{-3} diluted inocula. He did not, however, systematically compare different seed dilutions during numerous successive passages. Lundbäck states in another connection that the mumps virus strain used by him grew irregularly in the allantoic cavity although the inoculum was diluted 10^{-2}. Might this mean that even with 10^{-2} diluted inocula a fluctuation of titres occurs resembling that seen in the UP-series. It will be remembered that in the case of some of the allantoic fluids clearly better yields of hemagglutinins were obtained in the present work with inocula diluted 10^{-4} or more than with inocula diluted 10^{-2}—10^{-3} or less (fig. 20, table 3). The results did not show, however, whether such a situation can arise if *successive* passages are performed with 10^{-2}—10^{-3} diluted inocula as recommended by Lundbäck. Further work on this problem is desirable.

In the present investigation the inoculum was generally diluted 10^{-4} in the standard passages, in some experiments 10^{-5} or 10^{-6}. The number of these standard passages performed in the course of over one year totals about 150. Apart from a few of the first allantoic passages of Habel strain, fairly constant and high hemagglutination titres developed in the pooled allantoic fluids (average 6 eggs) during all standard passages of the four virus strains used. Thus, in the author's opinion, 10^{-4}—10^{-5} diluted allantoic fluids containing about 10^3—10^5 EID_{50} per inoculum, are to be regarded as suitable seed doses for example in the preparation of antigens and vaccines.

2. »Insensitive» Eggs

Egg variation usually had no major adverse effects on the yields of hemagglutinins in the standard passages, nor did it hamper the infectivity titrations. The single exception was a period of about two months during which »insensitive eggs» occurred to such an extent that infectivity titrations could not be properly

performed. The author's experience as regards this »bad season» is very much in line with those of other observers (61, 14), indeed the »bad season» occurred at the same time of the year as it had done in England several times (14).

Hoyle's recent studies in England (90) regarding the nucleotide composition of influenza virus ribonucleic acid deserve attention in this connection. This nucleotide composition in fact is very different in the winter months as compared with the other seasons, evidently owing to seasonal differences in the eggs. Studies of this kind concerning variations in the composition of eggs might perhaps throw additional light on the poor multiplication of mumps virus in some eggs. If these causes of the egg variation could be further elucidated, more light may also conceivably be shed on circumstances of more general interest associated with the mechanism of virus multiplication.

The writer thinks that an occasional »insensitivity» of the eggs in any case may greatly hamper all studies in which mumps viruses are cultivated in the allantoic cavity of chick embryos.

3. Nature of the Phenomena Appearing during UP-series

In the majority of the UP-series performed, hemagglutination was the only criterion of viruses. The typical fluctuation of titres was observed in all UP-series, and in different UP-series with the same virus strain and the same incubation time the phenomenon was reproducible in much the same form.

Analysis of the nature of the phenomena appearing during the UP-series is rendered difficult by the fact that relatively little is known of the mumps virus and of its multiplication in embryonated eggs. Mumps virus has special features of its own, and for this reason it may be dangerous to apply information on the behaviour of other extensively studied myxoviruses, notably influenza viruses, as such to the mumps virus.

The titres of individual eggs could vary greatly with the decrease and increase in hemagglutinin titres during the UP-series. It is obvious that the hemagglutinin titre of a pooled allantoic fluid may then give a very poor picture of the yield of hemagglutinins. On the one hand, when allantoic fluids are pooled, the inhibitor

in the fluids derived from the negative eggs tends to depress the hemagglutinin titres; on the other hand a few high-titred allantoic fluids, differing greatly from the majority of fluids, tend to raise the titres. The development of such high hemagglutinin titres in a few eggs during a UP-series, while the yields were very low in the majority of eggs, was a phenomenon frequently observed (figs. 9, 10, 11 B, 12 B, 11). A similar phenomenon appeared in Henle & Paucker's studies (81) dealing with the interfering capacity of inactivated influenza viruses: if the interfering dose was small, high virus titres developed in a small percentage of the eggs, while at the same time virus multiplication was greatly inhibited in the large majority of the eggs. The phenomenon appearing during UP-series of Rift Valley Fever virus (122) is possibly to some extent analogous: in the great majority of the mice the incubation time distinctly lengthened during the UP-series, whereas the incubation time was surprisingly short in a small percentage of the mice.

According to Horsfall (88), large amounts of hemagglutinating but noninfective viruses form in the allantoic fluids if the multiplicity of the inoculum is 3 or more. This observation could not be confirmed in the present work: in the *first* undiluted passage of Enders strain the EID_{50}/HA value of the yield, up to the 5th day at least, was regularly of the same order as the corresponding value for ST-viruses, although the inoculum contained about 10^9 infectious doses (figs. 14, 15, 17). From the present author's results it thus seems that a high ST-virus multiplicity is not alone capable of producing at any rate large amounts of virus material having a distinctly lower EID_{50}/HA value than ST-viruses. All the experiments concerned were made with the Enders strain. There are no statements in Horsfall's report as to the strain used for the observations. Possibly the different findings may be due to differences in the strains used.

The decrease in the EID_{50}/HA ratio of mumps virus preparations during serial undiluted passages (figs. 14, 15, 16, 17) resembles the von Magnus phenomenon (118). In both cases the EID_{50}/HA ratio only decreases to a minimum after several undiluted passages and then again increases in later passages. The decrease of this ratio during serial undiluted passages of mumps virus can be explained as being due to either inactivation of ST-viruses *in ovo*

or to the formation of incomplete mumps viruses analogous to incomplete influenza viruses. In favour of the latter possibility, in the author's opinion, is the fact that the EID_{50}/HA ratio does not begin to decrease until after several successive undiluted passages and the observation that the same number of passages were required for this ratio to decrease irrespective of whether the incubation time in the individual passage was 3 or 5 days (figs. 15, 17, and pages 59—60).

Typical of the von Magnus phenomenon is the fact that during the UP-series the yields of hemagglutinins decrease considerably less than the yields of infectious virus (118). Thus, although the yields improve by diluting the inoculum, this is no case of true interference, since all viral activities are not inhibited, at least not to the same extent (45). In UP-series of mumps virus the situation seems to be different. Both infectivity and hemagglutinin titres dropped greatly. The exact EID_{50}/HA value could not then be determined, because the yield of hemagglutinins always fell below the level of detectability. Concentration tests, however, showed (pages 53—56) that the production of both infective virus and hemagglutinins had obviously decreased to the same extent. It also seems that the decrease in hemagglutinin titres cannot be accounted for as a »masking phenomenon» due to an increase of inhibitors (pages 41—42 and 44). Moreover, the CF titres also decreased simultaneously (fig. 4). The propagation of ST-viruses inoculated at the same time as the UP-allantoic fluid or a few hours later, was inhibited (table 3), and on the other hand, the yields were improved by diluting the inoculum (figs. 18, 19, 20, tables 3 and 4). All these data thus indicate that interfering material emerges in the allantoic fluids during successive undiluted passages of mumps virus.

The interference phenomena associated with UP-series of mumps virus in the allantoic cavity of chick embryos bear a resemblance to the interference phenomena observed during UP-series of Rift Valley Fever virus in mice (122) and UP-series of vesicular stomatitis virus in monolayer tissue cultures of chick embryos (30).

It was shown above (page 69) that, in relation to the hemagglutinin titres, the interfering capacity of the UP-allantoic fluids was at least one thousand times stronger than that of Δ ST-

viruses, and on the other hand, the multiplicities of the virus particles (calculated on the basis of the HA titres) required for inducing interference could evidently be considerably less than 1. Seeing that the HA and CF titres of the allantoic fluids during the UP-series were practically in accord (fig. 4), it thus seems that the interfering capacity of the UP-allantoic fluids also correlates poorly with their complement fixing activity. The author feels that these circumstances might be best accounted for by assuming that the UP-allantoic fluids contain interfering material which is not capable of hemagglutinating and does not possess complement fixing activity. This interfering material could scarcely be a degradation product of ST-viruses, for the following reasons: 1) After losing their infectivity, mumps viruses (Enders strain) retain their hemagglutinating activity *in vitro* at +35°C for several months (26). The CF activity is obviously even more stable (9, 103, 5). 2) The

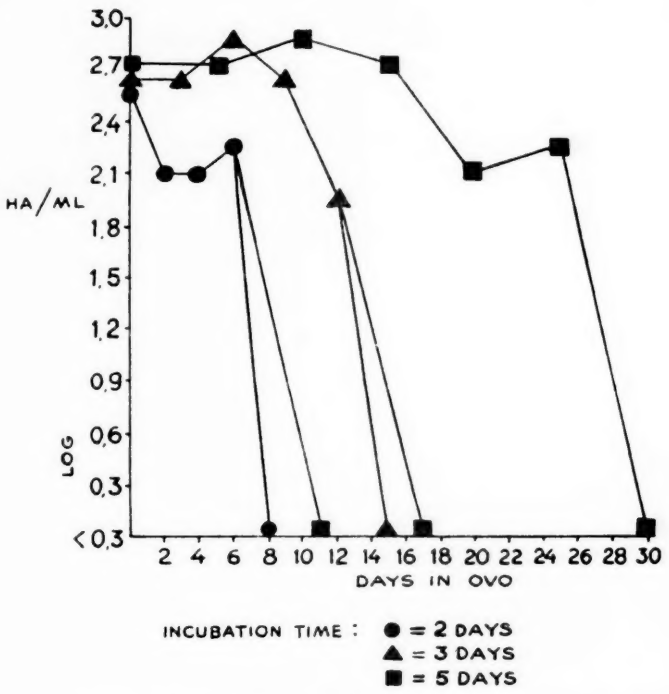

Fig. 21. — *Enders strain.* Hemagglutinin yields during UP-series with incubation time of 2, 3 or 5 days in each passage.

typical UP-series of Enders strain performed using incubation times of 2, 3 and 5 days are presented in figure 21. It shows the tendency seen also in other corresponding experiments: the shorter the incubation time in the individual passages, the less is the number of passages required for interfering material to develop in sufficient amount to depress the titres. If the interfering material were a degradation product, the opposite situation would be expected. The interference phenomena described above call to mind the interfering agent described by Isaacs *et al.* (94, 95, 96), the so-called interferon, which is produced by the interaction of a large inoculum of inactivated influenza viruses and chick chorioallantoic membranes *in vitro*. This interfering agent seems to lack other known viral activities. It has been assumed to be an intermediate product of influenza virus synthesis — possibly a mediator of viral interference.

SUMMARY

It was the purpose of the present work to study mumps virus multiplication in the allantoic cavity of chick embryos during serial passages using undiluted (UP-series) and 10^{-4}—10^{-6} diluted (ST-series) inocula.

ST-series. — About 150 standard passages were performed in the course of over one year. In the case of all four virus strains studied, the yields of hemagglutinins (HA) in pooled allantoic fluids were fairly constant during the standard passages. The greatest number of experiments were made with the Enders strain. Its infectivity titres (EID_{50}) in allantoic fluids ranged from about 10^9 to 10^{10}. The average EID_{50}/HA value was $10^{6.7}$.

In the great majority of the individual chick embryos the hemagglutinin titres varied fairly little in one and the same passage. In a small percentage of the eggs hemagglutinins did not develop in measurable amounts. Such negative eggs were very frequent during about two months in the course of the experiments, which made it almost impossible to perform infectivity titrations during this period. The causes of and disadvantages due to these egg variations are discussed.

UP-series. — The hemagglutinin titres of all four strains fluctuated greatly during successive undiluted passages: they kept falling below the level of detectability, rising again to the original level. In the first few undiluted passages the levels of hemagglutinins were always as high as in the standard passages. When the incubation time was 5 days in the individual passages, the titres of the Enders strain generally dropped in the sixth passage. If the incubation time in the individual passages was shorter, 2 or 3 days, the titres already decreased in earlier passages. UP-series performed in parallel showed that the hemagglutination titres of Habel strain invariably decreased in earlier passages than those of Enders strain.

The CF titres of the allantoic fluids fluctuated in the UP-

series in the same way as the hemagglutinin titres. The nonhemagglutinating allantoic fluids also never displayed any complement fixing activity. All nonhemagglutinating allantoic fluids studied, however, contained at least 10^4 EID_{50}. Such nonhemagglutinating allantoic fluids were not more active inhibitors of the hemagglutination by ST-viruses than were normal control allantoic fluids. Storage of these nonhemagglutinating allantoic fluids at +37°C was never found to render them hemagglutinating.

The hemagglutinin titres of individual eggs varied greatly during the UP-series, more than during the parallel standard passages.

In the first few undiluted passages the EID_{50}/HA ratio in the allantoic fluids was of the same order as in the standard passages, *i.e.* about $10^{6.7}$. The EID_{50}/HA ratio decreased distinctly in the fourth undiluted passage (Enders strain) irrespective of whether the incubation time in individual passages was 3 or 5 days. In individual UP-series the EID_{50}/HA value was found to be maximally about 1.5 to 2.5 log lower than the corresponding average value for standard viruses. When the hemagglutinin titres became negative in the UP-series, the EID_{50}/HA ratio at the same time increased to approach the corresponding value for ST-virus preparations.

The UP-allantoic fluids derived from the passage preceding the drop in hemagglutinin titres in the UP-series were capable of interfering against the multiplication of ST-viruses. The interfering capacity of such UP-allantoic fluids was definitely stronger than that of ST-virus preparations inactivated *in vitro* at +35°C in relation to the hemagglutinin titres. When such UP-allantoic fluids were used as inocula the yields increased with dilution of seed. An »autointerference» of this kind occasionally still appeared when the diluted inoculum contained only less than 10^{-1} HA units.

The adverse effects of undiluted inocula are dealt with from the point of view of practical virology.

The phenomena appearing during the UP-series are compared with the von Magnus phenomenon.

The interference experiments suggest that during UP-series a material develops in the allantoic fluids which has no hemagglutinating or complement fixing activity, yet is capable of inducing interference. This interfering material can scarcely be a degradation product of viruses.

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